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Experiments in the large-scale culture of the larvae of *Ostrea edulis* L.

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INTRODUCTION

The culture requirements of oyster and other bivalve larvae have been the subject of numerous studies (see review by Loosanoff & Davis, 1963; Walne, 1964*a*). The resulting knowledge has been applied at Conway to the development of a technique for rearing large numbers of oyster larvae, and this paper describes the data obtained from rearing 59 batches by a standard method over a period of two years (1963 and 1964). The data from these experiments give the average result that can be expected with this technique, and will provide a basis for future developments in larval rearing techniques. Because experiments of this nature cannot be quite identical, the results also give indications of the ways in which the method could be improved.

The satisfactory culture of oyster larvae depends on adequate supplies of sea water, algal food and larvae, and the methods by which each of these was obtained are described. While this work was in progress the requirements of the spat were also being investigated. This aspect has hitherto been neglected, but there is no doubt that improvements in this field could lead to a substantial increase in growth and survival in the period from spatfall to removal from the collectors. For this reason, only a brief section on the methods of dealing with the spat is included in this paper.

The work described was done in a set of laboratories specially designed for experimental work on the rearing and culture of shellfish. The accommodation includes a store and workshop, four small laboratories, a tank room for the maintenance of the breeding stock, an algal culture room and a hatchery for larvae. The unit is adjacent to the Mussel Purification Tanks, Conway, about four miles above the mouth of the River Conway.

METHODS

THE SEAWATER SYSTEM

Sea water was pumped from the edge of the river channel, close to the low-water mark of spring tides, by a cast-iron centrifugal pump through a cast-iron pipe into a 20 000-gallon outdoor concrete storage tank. Water of suitable salinity could only be obtained at around high water; it was standard practice not to fill the tank unless the salinity was at least 30°/$_{oo}$, but some difficulty was experienced when a period of wet weather coincided with a neap tide period.

It was found to be most important that the storage tank should be clean, and it became the practice to sweep and hose it out at every spring tide. In addition the walls were swilled down with a chlorine solution once a fortnight in summer and once a month in winter. A set of three 100-gallon linked fibreglass tanks in the laboratory roof were kept automatically filled from this storage tank by a plastic-lined pump controlled by a float switch in one of the roof tanks. For pipework from the concrete storage tank onwards, polythene was used, and throughout the laboratory polythene or glass. Adjacent to the storage tanks in the loft there was a further fibreglass tank fitted with three 3-kW immersion heaters covered with 'Vitreosil' and controlled by a thermostat. This tank supplied warmed sea water through standard polythene pipes to the tank room and the hatchery. Sea water at ambient temperature was supplied to all rooms.

The salinity of the water in the laboratory system was recorded on most days by observing the specific gravity and temperature of a sample collected from the tank room; it was usually in the range 31-34°/$_{oo}$. The data covering the two seasons of work described in this paper are given in Figure 1.

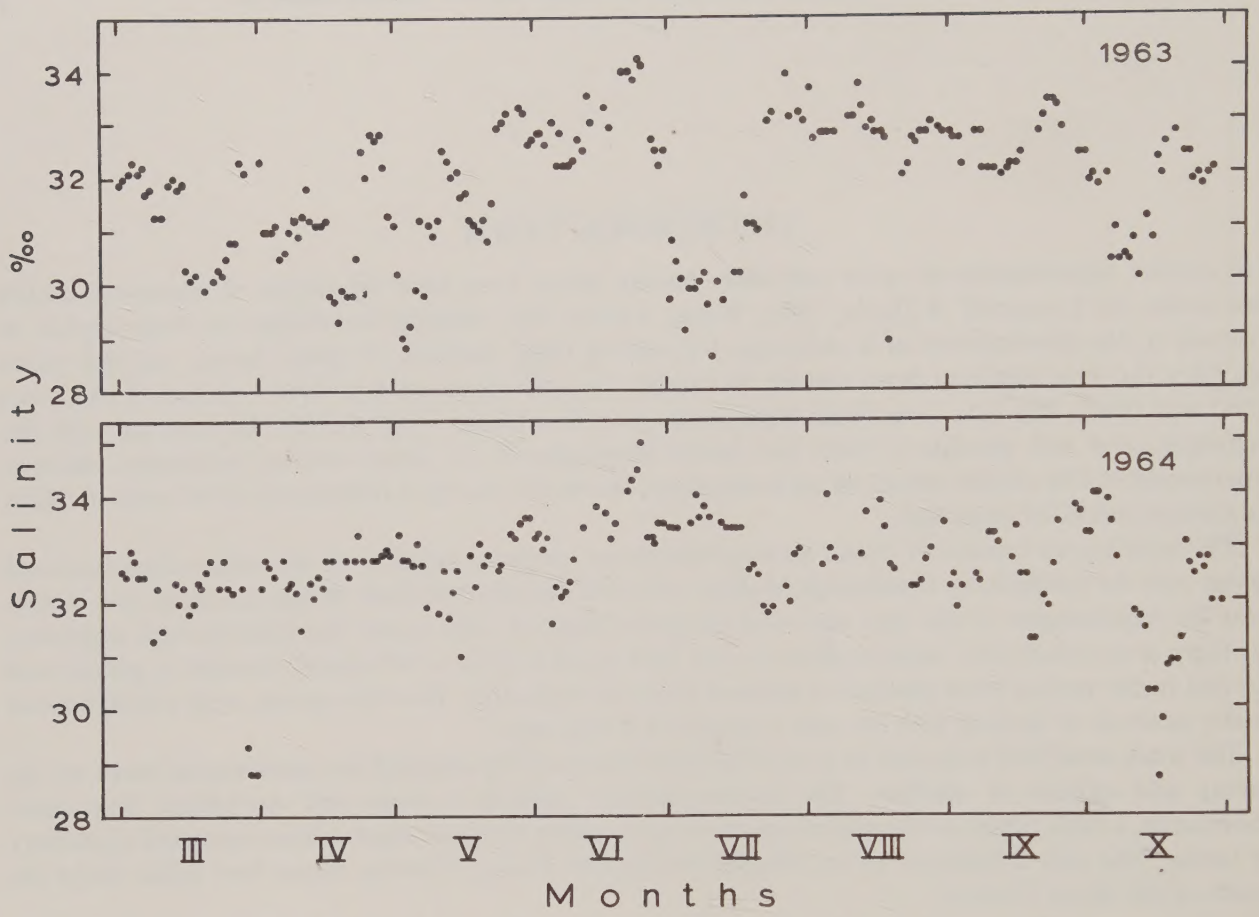

Fig. 1. *Daily salinity records of the laboratory seawater supply.*

LARGE-SCALE ALGAL CULTURES

Rearing experiments of the type detailed in this paper require relatively large quantities of uni-algal culture over long periods, and the guiding principle in evolving the system described below has been to develop a method which will give as high a yield as practicable and yet require only a short time for servicing each day. No attempt was made to rear bacteria-free cultures, because this would increase the complexity of the apparatus; moreover, some comparative feeding experiments (Walne, 1963) in which larvae were fed on cultures of *Isochrysis* and *Phaeodactylum* with and without bacteria showed no consistent benefit from using either food in a bacteria-free condition.

In the early stages of the work *Phaeodactylum* was cultured in a simple apparatus which gave good results, particularly in the spring and early winter. The culture vessels were glass tanks holding 12 litres of medium and these stood in aquaria through which cooling water flowed. Continuous light was provided by pairs of 100-W filament lamps shining through opposite sides of the tank, and the contents were briskly stirred by bubbling with air enriched with CO_2. The proportion of CO_2 was adjusted to control the pH at about 8·0.

The medium was sea water, enriched with 1 ml per litre of a stock solution, the formula for which is given in Table 1. The sea water was sterilized in three stages: after an initial passage through a sand filter, it was pumped through a ceramic pressure filter which retained particles down to about 3μ in size; from the pressure filter it passed to a glass storage tank where it was kept until required, and here a 30-W ultra-violet strip light over the top of the tank prevented growth of contaminants and gave some further

sterilization. This system was satisfactory during the winter and spring but not during the summer, when the most serious contaminant was a colourless flagellate, *Monas* sp., which would ingest the *Phaeodactylum* cells and rapidly bring about the collapse of the culture. *Monas* has been found elsewhere to be a serious pest in algal cultures set up with only partially sterilized media (Raymont & Adams, 1958).

Table 1. *Stock enrichment solution for* Phaeodactylum *culture. 1 ml is added to each litre of sea water*

E.D.T.A. (Sodium salt)	3·36 g
$FeSO_4\ 7H_2O$	2·78 g
$NaH_2PO_4 2H_2O$	30·0 g
$NaNO_3$	300·0 g
$MnCl_2\ 4H_2O$	4·7 g
Distilled water	to 1 litre

The cultures were usually harvested daily by dipping out 4 litres and replacing with fresh medium. A satisfactory check on the cell density was given by measuring the depth at which a disc of white Perspex became invisible; the cultures usually contained more than 20 000 cells per µl when harvested. Figure 2 gives the yield of a unit of 6 tanks for 90 consecutive days from 28 January 1959; the sharp reduction at 40-42 days demonstrates the results obtained in apparatus of this kind when an infection of the whole system occurs.

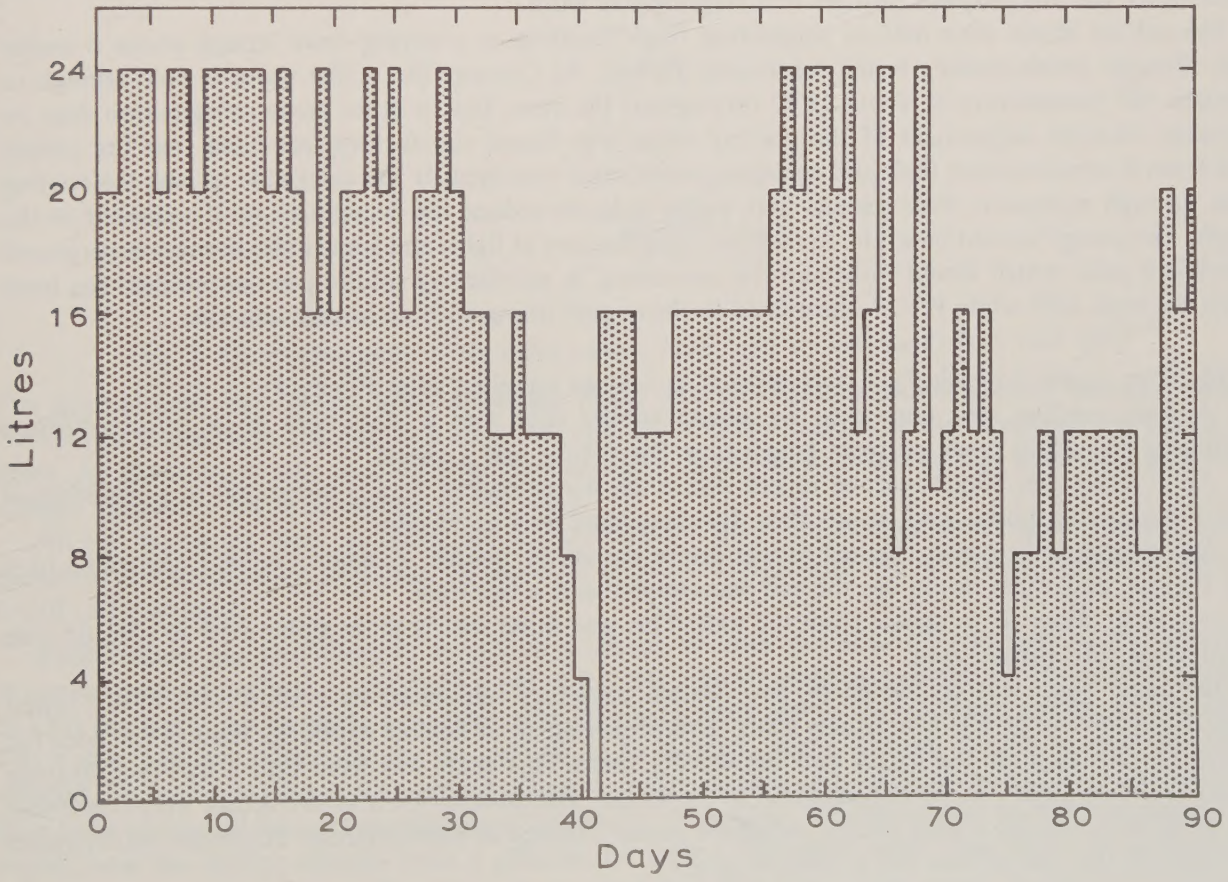

Fig. 2. *The daily yield in litres of* Phaeodactylum *culture from a unit of six culture tanks for a period of* 90 *days from* 28 *January* 1959.

Dunaliella tertiolecta can be cultured in this type of apparatus but not *Isochrysis galbana*, because the latter succumbs too rapidly to contamination. Since uni-algal cultures of *Isochrysis* were required in large quantities to feed the larvae and parent stock, the more elaborate apparatus described in the next paragraphs was evolved and is now used for all the large-scale algal cultures. A general view of the apparatus is shown in Plate 1. A diagram of the arrangement of tubes and stirrer in the culture vessel is given in Figure 3.

CONSTRUCTION, MAINTENANCE AND YIELD OF A LARGE-SCALE ALGAL CULTURE APPARATUS

Apparatus. The basic culture vessels were 20-litre flasks, the whole apparatus being made up of 14 such vessels arranged in two rows of seven, separated by a central row of three fluorescent strip lights above which were the culture-medium and air distribution lines. The flasks were of hard glass with a flared neck ending in ground glass. This type of neck is standard in a wide range of flasks and piping, and in conjunction with a backing flange and suitable insert and gasket, it allows a steel plate to be bolted over the mouth of the flask. On this plate was fixed the central stirrer bearing and the seals for four glass tubes. The arrangement of the stirrer and the use of glass vessels closed by a stainless steel plate is a simplified version of the method developed for the continuous culture of bacteria by Elsworth *et al.* (1956, 1958). The stainless steel stirrer shaft ended in a four-bladed stainless steel paddle. All the metal parts were made from EN 58B stainless steel, which has been found to resist corrosion by sea water, to be non-toxic for this purpose, and yet relatively easy to work.

The culture flasks were held in supporting rings standing in a copper-lined trough about 6 inches deep, through which cooling water continually flowed. At Conway the mains water is cold enough to maintain the temperature at about 18°C throughout the year, but in other areas refrigeration may be necessary. Manual adjustment of the cooling water was found satisfactory, provided that the supply came from a constant-level tank and a spigot control cock was used. It was advisable to pass the cooling water through a pressure filter packed with paper pulp, to reduce the accumulation of sediment in the trough. The trough should be white, to increase the reflection of light, and some difficulty was experienced in finding a paint which would withstand the conditions. A satisfactory solution to the problem has been to line the bath with white P.V.C. sheet fixed to the copper lining with an impact adhesive.

The four outlets to each flask were utilized as follows (labelled as in Figure 3):

(a) A *fresh medium tube* connected by silicon rubber tube and a glass cock to a culture-medium distribution manifold made up from $\frac{5}{8}$-inch bore Pyrex tube and T-pieces.

(b) An *aeration tube* which reached to just above the stirrer paddle. It was connected to a manifold of glass T-pieces and tubing, and the air flow was controlled by a screw clip.

(c) A *harvesting tube* which reached to just above the stirrer paddle. A steam line was connected to it by a T-piece, and when not in use the end was capped with a test tube.

(d) A *vent* to allow air to escape from the unit. This was fitted with a glass globe used as a liquid trap and plugged with cotton wool.

The stirrer shaft was connected by two flexible couplings, separated by a short rod, to a 3-jawed chuck cut in the end of a $\frac{1}{2}$-inch driving shaft. This arrangement of flexible couplings reduces the need for extreme care in aligning the stirrer and the driving shaft. This shaft was fixed into a ball-race bearing and carried a vee-pulley at the opposite end to the chuck. The pulleys of a line of cultures were connected by a series of V-belts to a $\frac{1}{2}$-hp electric induction motor running at 1420 rev/min. The speed was reduced by pulleys so that the stirrers ran at 200 rev/min.

The two rows of cultures were continuously lit by a bank of three 125-W fluorescent tubes placed in the centre between the two rows of cultures and by a single reflector tube on the outside of each row.

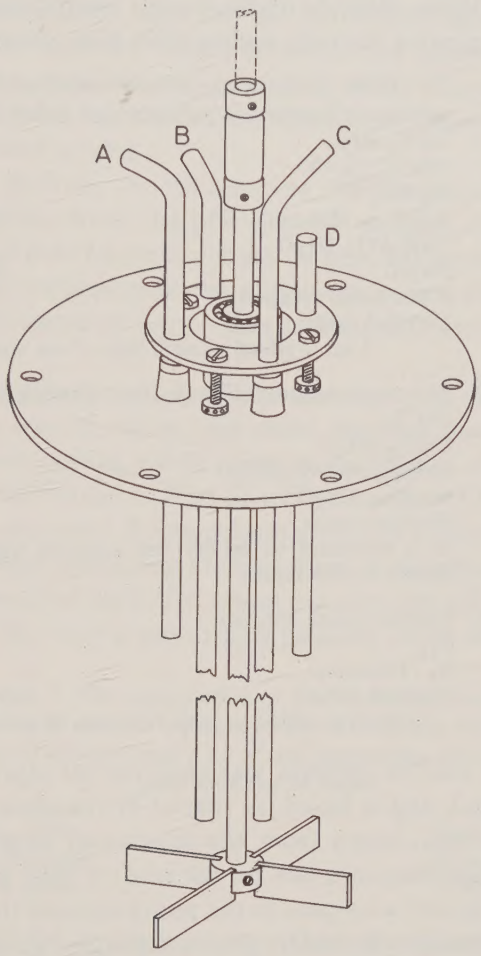

Fig. 3. *The closing plate of the algal culture flask, with its attached stirrer and tubes.*

The general light flux, taken inside an empty flask, was 250–300 foot-candles facing the three tubes, 130–200 foot-candles in the opposite direction, and 100–130 foot-candles in the directions not facing the lights. An experiment with an additional bank of lights on the outside showed that extra light gave only a slight increase in the division rate of the algae.

All the harvesting lines, air lines and culture-medium distribution lines were connected to a steam generator for sterilization purposes. A domestic pressure cooker with the lid drilled to take a tap was found to be suitable.

Each culture was continuously aerated with about 50 litres per hour of a mixture of air and CO_2 in appropriate proportions to maintain a pH of 7·5–8·0. The air acted as a carrier for the small quantities of CO_2 required, and by using a suitable air/CO_2 mixture the pH of the sea water could not fall below 7·5; it was therefore impossible for the cultures to be harmed even if removal of CO_2 by photosynthesis stopped. The addition of pure CO_2 would require a precise method of regulating the gas flow which would have to be controlled by a pH sensing probe inserted in the culture. The air was drawn from the laboratory supply, and the carbon dioxide from a cylinder through a reduction valve. The flow rate of each gas supply was controlled by a needle valve and measured by a manometer flow meter; in this, the gas passed through a short length of capillary tubing and a manometer connected across the capillary measured the pressure drop in the tube. This was proportional to the flow rate. Experience showed the flow rates of the

two gases which reduced the pH of normal sea water (about 8·0) to 7·5. After mixing, the gases were dried by passage through a calcium chloride drying tower, filtered through a tube plugged with cotton wool, and finally sterilized by passing through a glass fibre filter which retained particles down to 0·5μ.

Table 2. *Stock enrichment solutions for the culture of* Isochrysis galbana *and other algae*

1. $FeCl_3.6H_2O$ .. .. 2·60 g
   $MnCl_2.4H_2O$ .. .. 0·72 g
   H_3BO_3 .. .. 67·20 g
   E.D.T.A. (Na salt) .. .. 90·00 g
   $NaH_2PO_4.2H_2O$ .. .. 40·00 g
   $NaNO_3$ .. .. 200·00 g
   Trace metal solution .. .. .. 2·0 ml
   Distilled water .. .. to 2 litres
   1 ml is added to each litre of sea water

2. The trace metal solution has the following composition
   $ZnCL_2$ .. .. 2·1 g
   $CoCl_2.6H_2O$ .. .. 2·0 g
   $(NH_4)_6\ Mo_7O_{24}4H_2O$ .. .. .. 0·9 g
   $Cu\ SO_4.\ 5H_2O$ .. .. 2·0 g
   Distilled water .. .. to 100 ml
   It is necessary to acidify this solution with HCl to obtain a clear liquid.

3. Vitamin: stock solution
   B_{12} .. .. 10 mg
   B_1 (Thiamine) .. .. 200 mg
   Distilled water .. .. to 200 ml
   10 ml is added to each 100 litres of sea water

For convenience the same culture medium was used for all algal species. The composition of the medium used is given in Table 2, and is based on that of Provasoli *et al.* (1957). It was prepared in the following manner. Sea water was drawn from the laboratory supply and pumped directly without filtration to a polythene tank standing in a box on the roof. A float switch in the tank ensured that the correct quantity was pumped up and a by-pass to the pump allowed the nutrient salts and vitamins to be added. From the roof tank the medium flowed by gravity through a glass filter press, fitted with a sterilizing grade of cellulose-mineral mat, into a 100 litre reservoir flask. The filter had eight pads, 14 cm in diameter, and could be readily autoclaved. With an average head of about 36 inches 100 litres of medium took about 20 hours to pass through. The filter had to be taken down, fitted with new pads and autoclaved after 24 hours, because bacteria and protozoa can work their way through the pads after this time.

At one time the medium was heat-sterilized. This was done with a 2-kW immersion heater coated with "Vitreosil", inserted in the central neck of the flask, and a thermostat and stirrer in the side necks. The heater was controlled so that the water temperature did not exceed 90°C and the whole circuit was governed by a process timer. The cycle of sterilization took ten hours, of which about nine hours were required for the batch to reach 90°C. One of the disadvantages of this method was the time required for the medium to cool down. In addition, the long period at high temperature caused a substantial precipitation of salts from the water.

The medium was distributed to the culture flasks by a manifold connected to an outlet fixed to the bottom of the reservoir flask.

Operation and yield of the system. The cultures were harvested by siphoning 5 litres into a conical flask standing on a shelf alongside the cooling trough. The height of the shelf was such that when 5 litres of culture had come over, the levels in the flask and the culture vessel were the same. It was therefore not necessary to watch the flask during harvesting. When harvesting was finished the tube was shut off close to the flask with a screw clip and steam was blown through the harvesting line, which was then closed with a test tube. This prevented infection creeping back into the cultures. The cock at the bottom of the

medium reservoir flask was then opened, filling the distribution manifold, and sufficient medium was run into each culture vessel to restore the level. Each day the distribution manifold was steamed out, because infection had been found to travel from the culture vessels to the manifold, reproduce there, and infect other vessels. When not in use the manifold was connected to the filtered air supply. This ensured that potentially contaminated air could not leak back from the culture flasks. The reservoir flask and the air lines were steamed out once or twice a week.

Some cultures were examined each day for cell numbers and contaminants, and as a routine all cultures were examined at least once a week, when the pH was also checked. It was sometimes useful to keep counted cultures and it was found that *Isochrysis* could be kept for several days by holding the flasks at about 5°C in a refrigerator. Some settlement of the cells occurred but they could be shaken up and fed directly to larvae at 22°C without apparent harm. This technique is useful in smoothing out the supply of culture.

When cultures became contaminated, or if dead or non-suspended cells accumulated on the sides of the culture vessel and the culture lost its vigour, the vessel was first thoroughly cleaned and then, after re-assembly, sterilized by vigorously boiling a little water in the bottom of the flask. Cultures were always restarted from 'master cultures' maintained in 'Erd-Schreiber' medium (Table 3). These were maintained independently of the large-scale unit, and a 2-litre culture grown in a 5-litre conical flask was an adequate inoculum for a 20-litre culture vessel containing about 5 litres of the standard medium (Table 2). As the culture grew, further medium was added until after about five days the full volume of 20 litres was reached and stirring was begun. The first harvesting was usually possible about seven days after inoculation.

Table 3. *The composition of Erd-Schreiber medium. The soil extract is prepared by autoclaving together 1 litre of water and 1 kg of soil, decanting, sterilizing and allowing to stand*

$NaNO_3$	0·10 g
Na_2HPO_4	0·02 g
Soil extract	50 ml
Sea water	1 litre

Although the main purpose of the unit was to grow the large quantities of *Isochrysis* required in the experiments for the culture of larvae and spat, a number of other algae have been cultured and these are listed in Table 4, along with the cell densities obtained during regular harvesting. In the lower part of Figure 4 a detailed analysis is given of the yield of *Isochrysis* over a consecutive period of 100 weeks. The average weekly yield of a 20-litre *I. galbana* culture has been calculated from the mean of all cultures in progress in a given week, while the mean density is the mean of all the cell counts made during the week.

The average weekly yields over the whole period have been reduced to a comparable basis by expressing them as litres of culture containing 10 000 cells per µl. To complete the picture the upper part of Figure 4 gives the duration of each culture, plotted against the date on which it was started. Taking the period as a whole the average weekly results and the 95% confidence limits were:

Weekly yield from a 20-litre culture	29·22 ± 1·39 litres
Duration of culture	51·7 ± 5·6 days.

As an *Isochrysis* culture of this density contains about 0·1% of packed wet cells the output was about 4 ml wet packed cells a day. This yield compares favourably with those reported in the literature; for example, Wisely and Purday (1961) reported a maximum cell density of *Isochrysis* of $2{\cdot}2 \times 10^6$ cells/ml after 30 days of culture, compared with $10–15 \times 10^6$ cells/ml in the current work. Davis and Ukeles (1961) obtained 1·5 ml/day of packed wet cells from 5-gallon cultures.

Although substantial quantities are produced by this apparatus, losses of individual cultures by contamination are still a problem. It would certainly be better not to rely on one filtration as the only

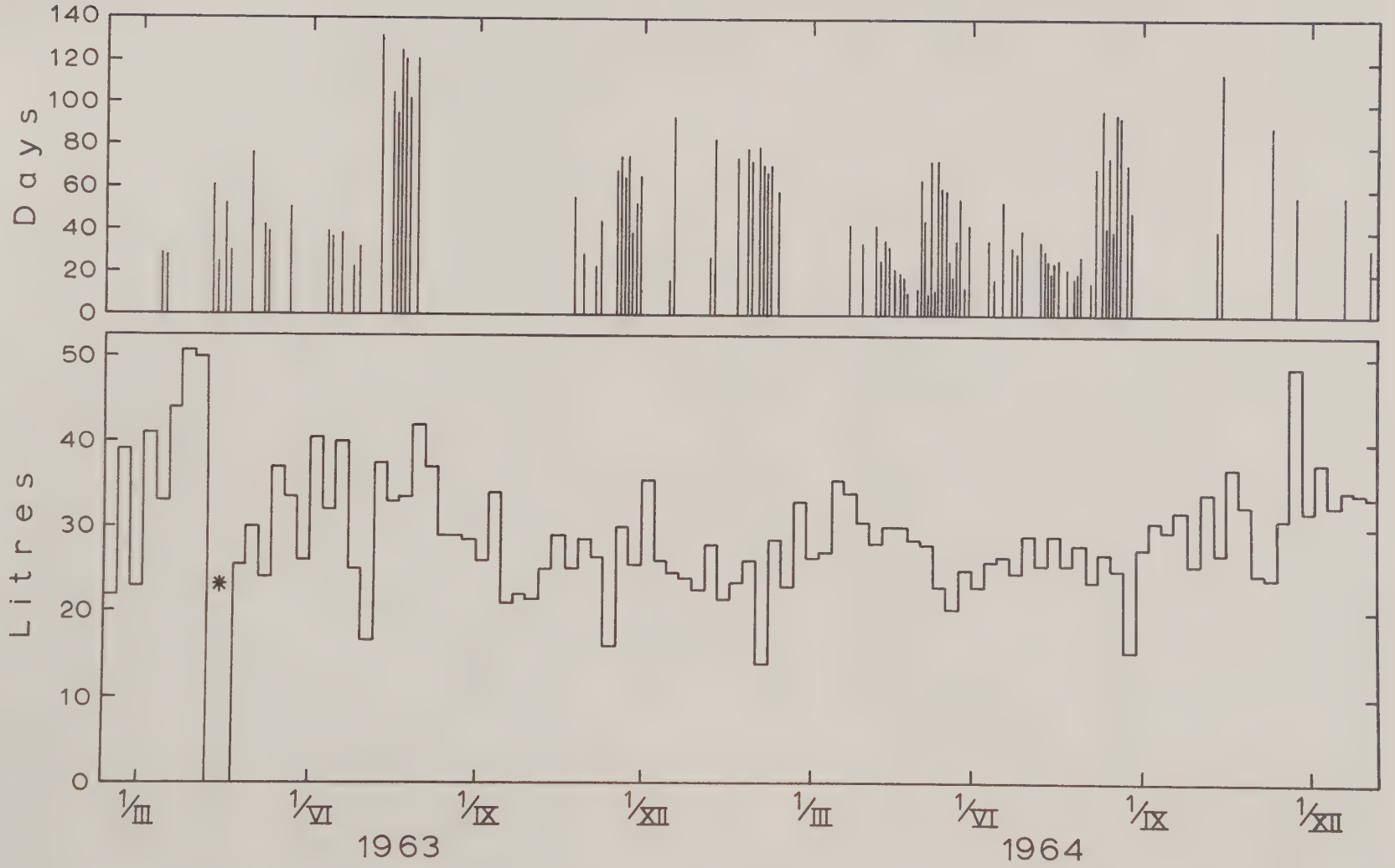

Fig. 4. *The yield of* Isochrysis galbana *from the algal culture unit for a period of* 100 *weeks from* 9 *February*, 1963. *The upper part of the diagram shows the duration of individual cultures, related to the date of inoculation. The lower curve gives the average yield from a* 20-*litre culture during successive weeks, expressed as litres of culture containing* 10 000 *cells/*µ*l.* ***Indicates a period of two weeks when no records of cell density were made.*

sterilization of the sea water. A single fault in filtration, however small, can result in a contaminant gaining access to most of the cultures, and once in the system there is a possibility of infection spreading. Davis and Ukeles (1961) used a ceramic filter permanently attached to each flask and by this method have maintained some species in uni-algal culture for over a year. This increases the complexity of the system, and unless pressure was available for filtration the rate of refilling the culture vessels would be slow. Any filters would require frequent autoclaving, since bacteria and protozoa can gradually grow through (Sykes, 1958).

The main contaminants have been ciliates and *Monas* sp., both of which eat algal cells. With a more intensive routine steaming of all pipework and the reservoir flask, this trouble was substantially reduced and in particular the spread of contaminants from one culture to another was checked. Small colourless flagellates 2–4μ in size appeared from time to time and have been shown to come through with the medium. These will co-exist in small numbers with *Isochrysis* and indefinitely with larger species.

On a number of occasions the cells of cultures of *Isochrysis* have become inactive, clumped together and failed to divide. This has been followed by the loss of the culture. Possible explanations include an accumulation of some type or types of bacteria, or that the culture medium may have become unbalanced in some way.

Table 4. *Species of algae grown in mass culture, and the cell densities obtained while they were being regularly harvested. The classification used is that given by Parke and Dixon (1964)*

	Cell density (No. per μl)
Bacillariophyceae	
Phaeodactylum tricornutum Bohlin	15 000
Haptophyaa	
Isochrysis galbana Parke	12 000
Cricosphaera sp. (Plymouth 156)	800
Chrysophyceae	
Monochrysis lutheri Droop	9 500
Pavlova gyrans Butcher	10 000
Prasinophyceae	
Micromonas pusilla (Butcher) Manton et Parke	20 000
Tetraselmis suecica Butcher	1 500
Chlorophyceae	
Dunaliella tertiolecta Butcher	2 500
Chlorella stigmatophora Butcher	16 000
Xanthophyceae	
Olisthodiscus sp. (Plymouth 239)	500

CULTURE OF THE BREEDING STOCK

The maintenance of the breeding stock of oysters in the best possible condition during maturation of the gonad, spawning and incubation of the larvae is an important factor in obtaining larvae which will subsequently grow well. The conditions required by the breeding adults have not been closely studied either during this work or elsewhere, and the methods described in this section have been developed by a process of judgment and experience rather than experiment. They are no doubt capable of considerable improvement but are described in some detail because of the successful results.

The tank room, 12 ft × 12 ft, had two bays each draining separately to the outside. The bays, separated from the centre strip by a concrete curb, were equipped with standard 2 ft × 1 ft × 1 ft iron-

framed aquaria standing on a wooden grid 4 inches above the ground and on a second tier 4 ft higher. The overflow of the lower tier of tanks drained directly on to the floor of the bay, while the upper tier drained into a gutter. One bay drained directly into the laboratory drainage system and the other into one of a pair of concrete tanks outside the building; this allowed the effluent from the second bay to be collected and chlorinated before it was discharged to the sea. The purpose of this arrangement was to permit the use of breeding stock from areas infected with pests which did not occur at Conway, notably *Urosalpinx*, *Crepidula* and *Mytilicola*. The first two pests are external and can be removed from the shell by careful scrubbing, although in practice it is impossible to ensure the complete removal of very small individuals. *Mytilicola* is an internal parasite and, although rare in oysters, it cannot be removed.

Iron-framed aquaria were originally used because they could be glazed with non-toxic materials, were fairly cheap, movable, and one end could be closed with slate through which an overflow hole was readily drilled. They have the disadvantages that leaks can start when they are moved, the glass is fragile and the iron frames corrode, although we found that they had a life of about ten years. For replacement, polythene tanks, which have none of these disadvantages, are now used.

The oysters were held at about mid-depth on a grid of glass rods hanging from cross-bars resting on the top of the aquarium frame (Plate 2). Sea water ran on to the surface at one end of the tank, and a circulation through the tank was ensured by arranging the overflow through a glass tube which reached to the bottom of the tank at the opposite end to the inflow. An additional advantage of this arrangement was that the newly released larvae, swimming upwards, were less liable to pass through the overflow.

The sea water was distributed from a manifold made up of screwed polythene fittings which allowed the system to be frequently taken down and cleaned. Over each tank there was a T-piece fitted with a piece of rubber tube and a screw pinch clip which controlled the flow of water into the tank. Each tank was stocked with 10–15 oysters with a mean whole weight of 60–80 g. A flow rate of 10–15 litres per hour was maintained through the tanks and, because this was certainly less than the water available to each animal in the sea, an attempt was made to improve the feeding conditions by continually enriching the water with algal culture. This was probably particularly important in the winter months when the natural food content of the water was low. Although, as will be shown later, the stock did not maintain a good condition under this system, the finding of Collier (1959) that the pumping rate of *Crassostrea virginica* is related to the food content of the water suggests that some food is necessary for the oysters to pump satisfactorily. This could be important when larvae are developing in the mantle cavity. *Phaeodactylum tricornutum* and *Dunaliella tertiolecta*, because of their ease of culture, have been the main species used, although recent unpublished work suggests that they may not be ideal food organisms.

Warmed sea water was delivered from the loft by a polythene pipe via a ¾-inch hard rubber ball valve into a header tank and from this was distributed to the aquaria. In the header tank the water was enriched by the addition of approximately 200 ml of algal culture every 15 minutes from an algal reservoir tank immediately above. This tank had a bottom outlet connected to a narrow-bore rubber tube which passed via an electro-magnetic pinch valve to the header tank. The opening of the pinch valve was controlled by a timing device based on an electric motor with a shaft speed of 1 rev/hour. Mounted on the shaft were four radial arms and these in turn came in contact with a bracket holding a mercury tilt switch. The length of time that the arms held the switch in contact was adjusted so that the algal reservoir emptied in 24 hours. Recently this arrangement has been modified by removing the header tank, connecting the distribution manifold directly to the seawater supply, and injecting the algal culture directly into the supply pipe with a peristaltic dosing pump. This is a neater arrangement which gives a more regular enrichment and increases the water pressure in the distribution system. It was found important that there should be a good fall in all the pipework, because the system was frequently operating above the outside air temperature, and oxygen coming out of solution tended to form air locks.

A series of cell counts made on the water entering one of the tanks during a 15-minute period when the first dosing system was in operation shows the degree of enrichment obtained. The algal reservoir had

been filled with 5 litres of *D. tertiolecta* and 15 litres of *P. tricornutum* culture, and the diluted culture was feeding 13 tanks at a total flow rate of about 130 litres per hour.

Time in minutes	*Cells per µl*	
	P. tricornutum	*D. tertiolecta*
0	43	10
3	55	10
7	48	7
11	45	3
15	50	1

Oysters have a substantial oxygen consumption, and tests made on the inflow and outflow water showed that in a tank holding 10 oysters of 8–9 cm diameter each oyster was consuming 1–1·5 ml per hour.

Table 5. *The numbers of releases of larvae obtained from six different batches of breeding stock during successive 10-day periods, and the mean water temperature during the period up to the first release*

Number of oysters	97	101	101	50	100	47
Origin	Helford	West Mersea	Poole	Helford	Helford	Crouch
Date started	20 Feb. 1962	1 Mar. 1962	26 Apr. 1962	26 Mar. 1963	14 Jan. 1964	26 Feb. 1964
10-day period						
1						
2						
3		3				
4	1	4	1			2
5	1	7	4	2		2
6	4	3	4	2	3	1
7	4	3		2	5	3
8	5	1		1	2	1
9	0	1		4	2	
10	3	1			1	
11	3					
Total	21	23	9	11	13	9
Broods per 100 stock	21·6	22·8	8·9	22·0	13·0	19·3
Mean temperature (°C)	20·0	20·2	20·7	18·9	19·2	19·3

Before the sea water entered the tank room it was heated to about 20°C in a tank in the loft, by three thermostatically controlled 3-kW electric immersion heaters. It was found to be important that the tank room should be maintained at a constant temperature so that the water in the tanks had a constant cooling rate. In some preliminary experiments in an otherwise unheated room, infra-red heaters were found useful to control excessive cooling. In early experiments in which oysters were being conditioned for spawning, the water temperature was gradually raised a degree or so a day until it reached about 21°C, but the results obtained were no better than when the stock was transferred directly from water of 1–5° to 21°C. Stock brought into the tanks in the period January to March showed signs of shell shoot about ten to fourteen days later and the first liberations of larvae generally occurred after 30 to 40 days. Table 5 shows the numbers of broods of larvae obtained in successive ten-day periods from six batches of breeding stock, and the mean water temperature up to the time of the first release of larvae.

Table 6. *The average size and condition of two batches of oysters before and after being used for experimental breeding*

	R. Colne oysters		R. Blackwater oysters	
	22 Mar. 1961	31 May 1961	31 May 1961	19 July 1961
Diameter (mm)	77·3	80·3	65·9	65·6
Whole weight (g)	78·9	78·9	55·7	56·8
Dry meat weight (g)	2·09	1·12	1·40	0·86
Glycogen (g)	0·70	0·37	0·64	0·21
Condition Index*	110	58	115	71

* The condition index $= \frac{\text{mean dry weight of meat}}{\text{mean internal volume}} \times 1\,000$

Mortality of the breeding stock was usually about 5–10% over a 3–4 month period, but the failure to grow and the loss of meat weight in the following examples demonstrates how poor the feeding conditions were. Two batches of 100 oysters used for breeding stock in 1961 were each divided at random into two groups of 50, and the condition index was determined on one group when received and on the other group when breeding had finished 6–9 weeks later. The results (Table 6) showed that during this time both the dry meat weight and the glycogen content decreased by about half. Breeding populations in the field do not lose condition to this degree. It is probable that most of the larval production must have been from the food reserves present in the stock when it was first brought in, and it was therefore regarded as important to obtain stock which was in good condition. There is also some evidence that the number of larvae produced is influenced by the condition of the parent (Walne, 1964*b*).

When liberations of larvae were expected the tanks were examined several times during the day; their presence was easily seen, because they swim upwards and many collect as rafts against the surface film. When a liberation was first observed, it was recorded in the tank room diary and usually the time was noted. Some broods are liberated during the night but enough larvae remain for the occurrence to be observed up to 12 hours or more later. An analysis of this record (Table 7) for the three seasons 1962–64 showed that afternoon liberations were the most common; the reason for this is unknown, but at that time of day the oysters have been subjected to a period of vibration from the movement of people in the room and building. There is also likely to be more temperature variation by day than by night, due to the opening and closing of doors and the drawing-off of heated sea water for the larval cultures.

Sufficient larvae could usually be obtained by skimming off those near the surface. If the larvae were sparse or a large collection was wanted, then most of the water in the tank was filtered through a stainless steel sieve, with a square mesh of 104μ; it was found that a mesh of 124μ would let through some broods of larvae. A stainless steel filter on the overflow was tried but the results did not seem to be worth the effort necessary to keep it from becoming choked. It is probable that the main loss of larvae was caused by the filtering activity of the adult stock.

Table 7. *Numbers of broods of larvae liberated during different periods of the day. Those recorded as overnight were first seen between 0830 and 0930 hours; morning covers 0930 to about 1330 hours, and afternoon from 1330 to about 1800 hours*

	1962	1963	1964	Total
Overnight	13	8	10	31
Morning	27	7	8	42
Afternoon	50	26	24	100

CULTURE OF THE LARVAE

In this section the techniques used for culturing the larvae are described and this is followed by an analysis of the results obtained. In the discussion at the end of the paper some of the reasons for the adoption of the various methods are given and data from relevant experiments are cited.

Nineteen experiments were made in 1963 in the period May 10 to September 4. The 37 experiments in 1964 covered the period March 4 to September 9, but five of these experiments were prematurely ended due to a thermostat failure. The results of these five have been included where appropriate but they do not contribute to the yield of spat.

METHOD OF CULTURE OF THE LARVAE

After collection from the breeding tank the larvae were washed with filtered sea water through a sieve with a mesh of 185μ, which retained any larger debris, and collected on a fine-meshed sieve below. The shells of the larvae are strong enough for them to be washed with jets of water while on the sieve, without getting broken. A supply of filtered sea water was kept in a 2 ft × 1 ft × 1 ft glass tank standing on a shelf above the sink. A 30-W ultra-violet strip-light over the tank prevented the growth of bacteria and protozoa.

After washing, the brood was suspended in 2 litres of filtered sea water in a graduated cylinder, and, after thorough agitation with a perforated plastic plate attached to the end of a rod, three 1·0 or 0·5 ml samples were withdrawn, counted, and a random sample of 50 measured. Sufficient of the larval suspension to contain 100 000 larvae was then siphoned out of the cylinder while it was thoroughly agitated. In 1963 it was found that the number of larvae obtained by this method sometimes diverged rather widely from the 100 000 required, so in 1964 the number in the subsample was usually checked and adjusted if necessary. These larvae were placed in a polythene bin and the volume of water was made up to 25 litres in 1963 or 50 litres in 1964. In 1963 the volume was increased from 25 litres on day 0 to 50 on day 2 and to 75 on day 4; in 1964, 50 litres were used on day 0 as well as on day 2, and increased to 75 litres on day 4.

The water used in these rearing experiments was drawn from the warmed seawater supply (see above) and treated in the hatchery. It first flowed through an ebonite ball valve into a filter in which a one-inch layer of sand was spread on a nylon cloth laid on a sheet of perforated 'Perspex', supported about ½-inch off the bottom of a 12-inch deep fibreglass tank, with a bottom outlet. From the sand filter the water was pumped by a plastic pump through a ceramic pressure filter. This had a nominal pore size of 25–30μ and the 10-inch × 2-inch tubular element was held in an ebonite vessel. From the ceramic filter the water passed through an ultra-violet water sterilizer to a fibreglass reservoir. The ultra-violet sterilizer was a 44-W low-pressure mercury vapour discharge tube in a quartz envelope, and the water circulated between the envelope and an outer glass jacket. In 1964 the fibreglass reservoir was eliminated so as to reduce the surfaces liable to be responsible for bacterial contamination, and the water passed directly into the larval culture vessels.

At the beginning of the day warm sea water was run into the sand filter and the pump switched on as required. At the end of the day the sand filter was drained and the rest of the system flushed with fresh water. In 1963 chlorine solution was periodically pumped through the system, but in 1964 a solution of 3 ppm of chlorine in fresh water was passed through the system for five minutes at the beginning of each day. The ceramic filter elements were changed on most days and the sand filter twice weekly. The problem of reducing the bacterial flora in the seawater supply is dealt with in more detail on p.32.

The polythene bins used in these experiments had a diameter of 15½ inches at the base and 18 inches at the top; they were 23 inches high, and held 75 litres when full. Although opaque lids were available, it was convenient to use sheets of transparent P.V.C. as covers. The centre of the lid was drilled to take a glass aeration tube 6 mm in diameter which was connected by a rubber tube to a sintered glass aerator hanging centrally near the bottom of the bin. The rate of flow was controlled by a screw clip to give about 0·5–1·0 litres per minute. The compressed air was drawn from the laboratory supply and was filtered through cotton wool before use.

The temperature of the vessels could be regulated by the temperature of the room, but because of evaporation the room temperatures had to be maintained about 2°C higher that than required in the vessels. Some experiments were treated like this, but in the others temperature control was obtained through a 6-inch deep water bath. The bath was heated at one end by a domestic-type immersion heater and thermostat, and the water continually circulated by a pump. The whole assembly stood in an insulated room without windows, so that the experiments were in darkness except when observations were being made.

TREATMENT OF THE EXPERIMENTS

The day on which an experiment was set up was called day 0 and the subsequent days numbered 1, 2, 3, 4 and so on.

The larvae were fed with cultures of *Isochrysis galbana*, the preparation of which has been described in a previous section. On the days when the water was not changed the cell density in the culture was estimated by counting three 0·3μl samples with a haemacytometer; sufficient *Isochrysis* culture was then added to restore the cell density which, throughout the 1963 experiments, was maintained at 120 cells per μl. In 1964 this was reduced to 100 cells per μl and in the later stages of the experiments to 75 per μl.

Samples were obtained by thoroughly agitating the contents of the tank with a plastic disc and dipping out a small amount for the cell density count and a larger volume for estimation of the average size of the larval population. For the first two or three days, when the range of sizes was small, 50 larvae were measured; this was subsequently increased to 100. When eyed larvae appeared, the sizes of these were recorded separately. In 1963 the larval size was recorded daily but in 1964 it was recorded only on the days the water was changed.

The water was completely changed on days 2, 4, 5, 6 and 8, and thereafter on every alternate day. In 1963 the bacterial flora which had developed was examined by sampling the water immediately before

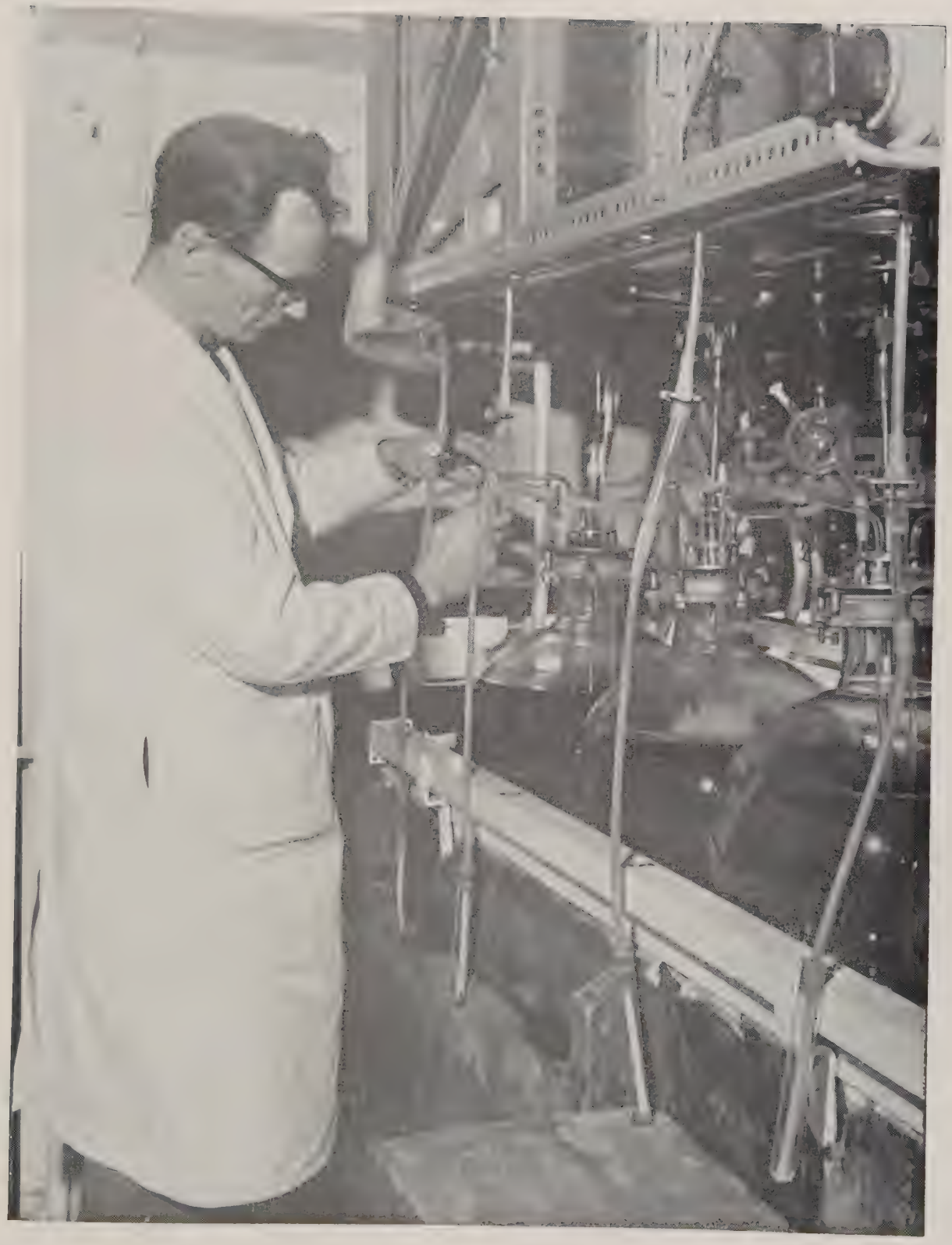

Plate 1. *The mass culture of the algal food.*
(*with acknowledgements to The Geographical Magazine, London*)

Plate 2. *The parent oysters kept in tanks of running sea water.*
(with acknowledgements to The Geographical Magazine, London)

changing. A 1-ml sample was withdrawn with a sterile pipette and diluted to 1 in a 100 by first adding to 9 ml of sterile sea water in a small bottle, thoroughly shaking, and then adding 1 ml of this to another 9 ml of sterile sea water. Two 1-ml samples of this dilution were then plated with 15 ml of Zobell's medium 2216 (Oxoid C 17239) and incubated for 48 hours at about 22°C. The number of colonies which had developed was estimated by counting 10 microscope fields and multiplying by the appropriate factor to give the number of colonies on a plate. In all of the 1963 experiments the density of the *Isochrysis* cells was also determined before the water was changed.

In all the experiments sufficient antibiotics were added at every water change to give a concentration of 50 i.u. penicillin G and 0·05 mg streptomycin sulphate per ml. A stock solution containing both these antibiotics was made in fresh water at such a strength that 1 ml was required per litre.

The water was removed from the bins by siphoning, the larvae being retained by a filter formed by a bag of bolting cloth fixed over a length of perforated ¾-inch polythene tube. This filter stood in the bin, and the water was removed through a siphon pipe inserted down the polythene tube. The filter was then washed to remove the adherent larvae, and the contents of the bin were washed into a bowl. After the larvae had been further concentrated by pouring the water through an 8-inch diameter stainless steel sieve with a mesh aperture of 124μ, they were washed through another of 4-inch diameter with an aperture of 295μ, which removed large particles of material. Finally the larvae were carefully washed by agitating them on a small-meshed sieve in a bowl of clean water and at the same time directing a jet of water on to them. At first the mesh of the sieve used for the final washing was 124μ; larger sizes were used as the larvae grew, until with the biggest larvae a mesh of 185μ was used.

Finally the larvae were drained and then washed into a 2-litre graduated cylinder for the purpose of estimating the number of live larvae. The contents of the cylinder were then put into a clean bin filled with freshly filtered water.

This procedure was followed at each water change except on day 4, when, after cleansing, the larvae were washed into a beaker containing 1 litre of sea water and sufficient of a commercial hypochlorite solution to give a concentration of 3 ppm of chlorine. The larvae remained in this for five minutes which was sufficient to kill most of the bacteria adhering to their shells. In 1964, the larvae were often given a rinse with 3 ppm chlorine during the routine water change. After chlorination, the larvae were well washed and their numbers estimated. On day 5 the estimation of the number of live larvae was usually omitted.

When eyed larvae were seen, two strings of five mussel shells were suspended near the surface. These shells were threaded on to stainless steel wire and separated by 1-cm lengths of glass tube. A daily index of the intensity of spatfall was given by counting the settlement on the shells. After the spat had been counted the shells were scrubbed to remove them and replaced.

When the test shells indicated that spatfall was beginning, collectors were added. These were similar in shape and size to the earthenware tiles used on the French oyster grounds, but were made from 5½-inch × 12-inch pieces of black matt-surfaced P.V.C. 0·030 inch thick. The collectors were tied in bundles with polythene twine. In the first few experiments bundles of eight collectors were used but these were found to be insufficient and in subsequent experiments were replaced by bundles of sixteen. The collectors were examined on most days and if it was judged that sufficient spat had settled, the bundle was changed. No experiment used more than thirty-two tiles. In some experiments the spat tended to settle more on one side of the collectors than the other and the collectors were then inverted for a few days. Settlement usually lasted 7-9 days.

The collectors stood on the bottom of the bin and the aeration system was modified to avoid bubbles continually moving across their under surface. A piece of ¾-inch polythene pipe with a few holes drilled in the side was inserted through the middle of the tile bundle. The diffuser was hung in the pipe, and the bubbles, drawing in water from round the bottom of the pipe and carrying it to the top, maintained a current of water in the bin.

When the collectors had been added the larvae were not sampled on the days when the water was not

changed, because of the difficulty of stirring the contents of the bin. The sampling of the bacteria was also discontinued at this stage.

RESULTS OF THE EXPERIMENTS

NUMBER OF LARVAE

The method adopted for the initial measuring out of 100 000 larvae was not very successful; the counts made on day 2 suggested that, on the average, in 1963 the experiments started with 110 000 larvae, with a range of 85 000 to 145 000, and in 1964 with about 96 000 with a range of 72 000-119 000 (one at 169 000). The major error was probably caused by the difficulty of siphoning out the correct volume while keeping the larvae in an even suspension. In both years the number of live larvae in the experiments (Figure 5) declined at an approximately uniform rate until days 10-12, when about 70 000 remained. After this the number dropped more rapidly, but even when spatfall had virtually ceased most experiments still had some 20 000 larvae which, although they did not develop further, were not obviously dead. The fact that the curve does not show a more rapid drop during days 4 to 6, when the water was being changed daily, suggests that most of the loss was due to death and that accidental loss during the water change played only a small part.

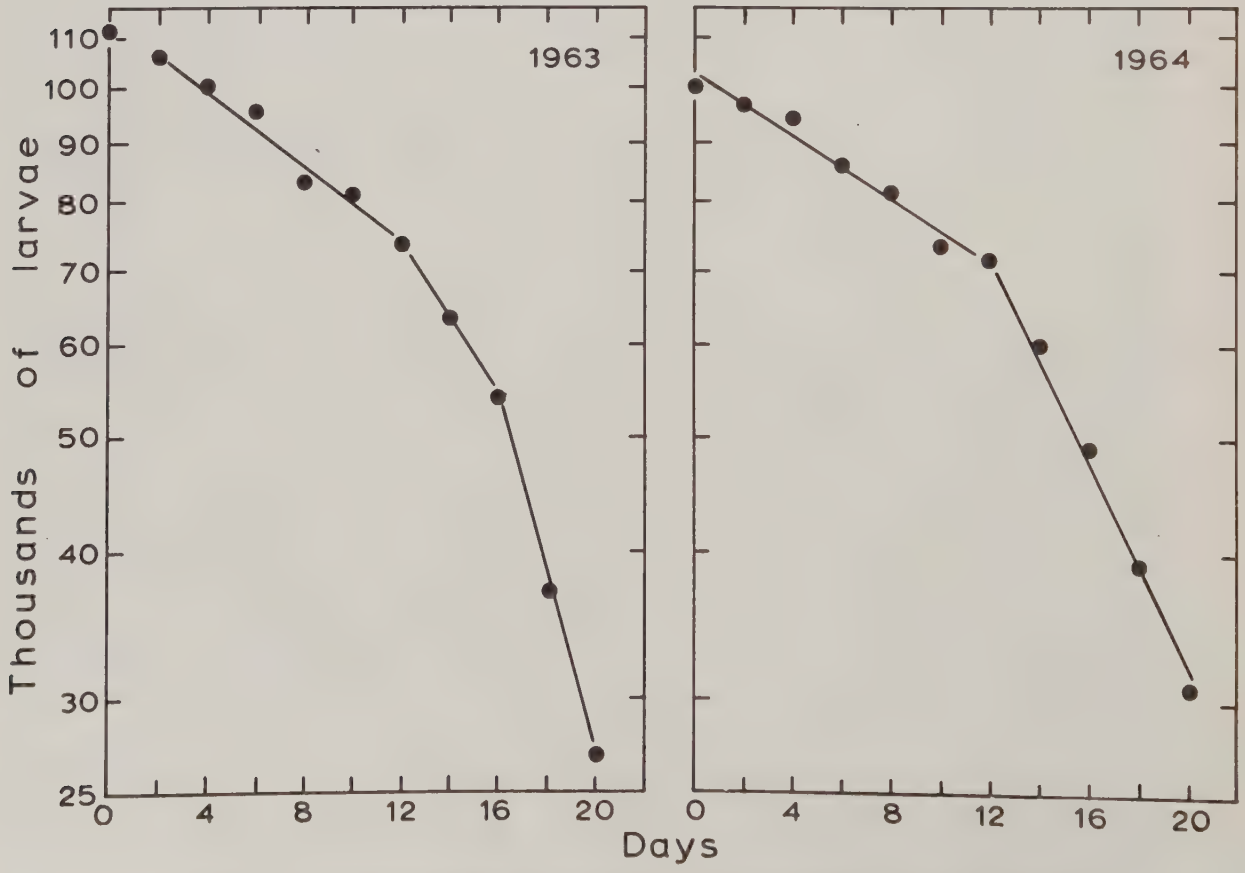

Fig. 5. *The mean numbers of live larvae in the experiments on successive days.*

In 1963 the average volume of water available to a larva increased from 0·23 ml on day 0 to 0·94 on day 10 (Figure 6A); this was caused by the loss of larvae and the increase in volume on days 2 and 4. Using data from Walne (1965), the mean dry weight of larvae per ml of water has been calculated (Figure 6B). This varied between about 2 and 4μg per ml, with a maximum value of 4.46μg of dry larva per ml on day 2, and over 4μg again after day 10. The original intention of maintaining about the same weight of larval material per unit volume was, therefore, only partially achieved. The situation was improved in the first part of the experiments in 1964 (Figure 6B), because of the increase of the volume from 25 litres to 50 litres in the first 48 hours, but there was still a considerable increase in the dry weight per unit volume as the larvae approached settling.

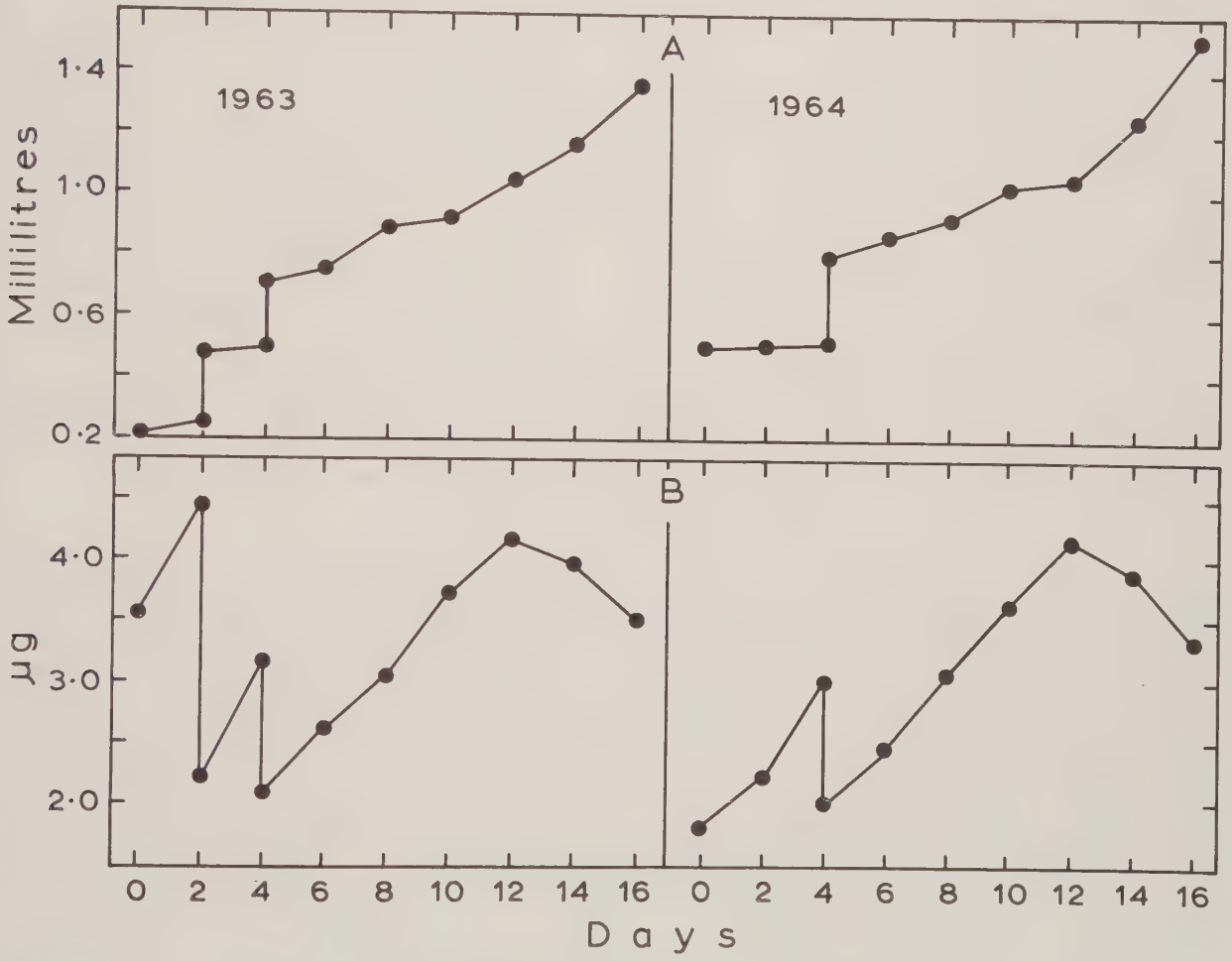

Fig. 6. (*A*) *The mean volume of water per larva and* (*B*) *the mean dry weight of larvae per ml of water in the experiments.*

The initial numbers of larvae in the 1963 experiments were between 83 000 and 145 000 in 25 litres and it is possible that this variation affected the initial growth rate, although the regression of the estimated number of larvae present on day 2 on the length increment obtained in the first 48 hours is not significant (Figure 7). For the rest of the time, and throughout 1964, there was no evidence of a relation between larval density and growth.

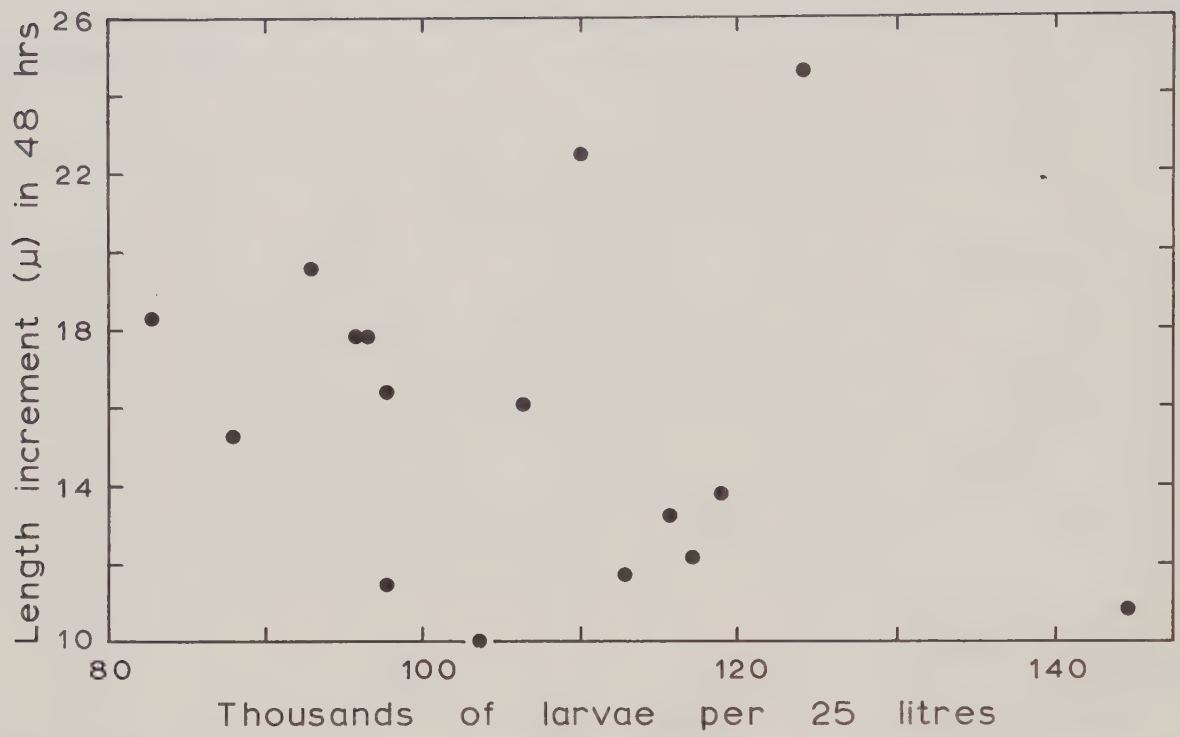

Fig. 7. *The relation between the length increment in the first* 48 *hours and the number of larvae in* 25 *litres in the* 1963 *experiments.*

GROWTH RATE OF LARVAE

Figure 8 shows the growth curves for the two years, calculated as the average mean size in each experiment; the range of means which occurred on each day is also given. In Figure 9 is shown the mean size distribution curve for each day from 0 to 10 in the 1963 series, calculated by summing the percentage size frequency distribution for each experiment and dividing the sum by the number of experiments providing data for that day. In Figure 10 the mean daily sizes have been related as 'Ford-Walford' plots; for the 1964 data the size at day n is plotted against the size at day n + 2, because the larvae were measured on alternate days.

The relation between the average size of the larvae and time is very nearly linear over the first 10-12 days (Figure 8), although individual experiments show considerable variation from this. In 1963 the main discrepancies were from day 0 to day 1 and from day 4 to day 5. In the first case the reason is probably that experiments were started over a variety of times during the day, depending on the liberation of larvae, so that the average time for growth was less that 24 hours. In the second example the period covered the 24 hours immediately after the larvae were washed in 3 ppm of chlorine, and this treatment may have caused a slight check in growth. This effect can be seen in the plot of length at day n against length at day n + 1 (Figure 10), where the increment at a mean length of 213·5μ (the length on day 4) was less than that obtained on other days. The 1964 data do not show these discrepancies, because the larval size was measured only every other day. The general trend of growth was very similar in the two years.

The range of sizes increased as the larvae grew (Figure 9) and by the tenth or eleventh day, when the most rapidly growing larvae were at settling size, the smallest were only 200-210μ. It is probable that these were not sufficiently robust to grow to, and survive, metamorphosis, and in the later stages of the experiment they were removed to some extent by using a larger-meshed sieve when washing the larvae.

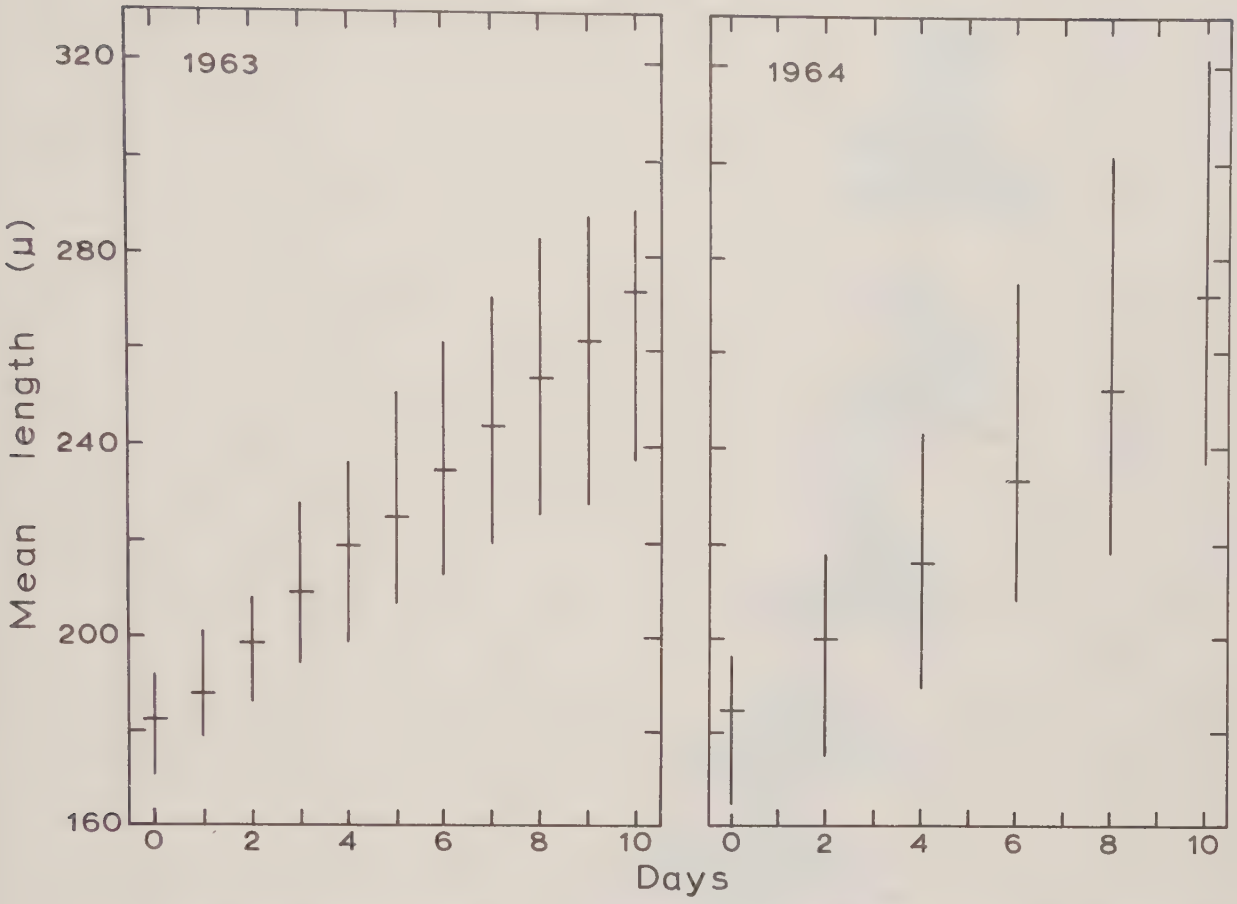

Fig. 8. *The mean length of the larvae in all experiments on successive days and the range of means obtained on successive days.*

EYED LARVAE

Eyed larvae were occasionally seen on day 9 and by day 10 they were present in 40% of the experiments, with an average of 10 000 in an experiment (Figures 11 and 12). Their appearance was sometimes delayed, and eyed larvae were not found in all experiments until day 14 or 15. On the average the peak number occurred on day 16, with 33 000 in 1963 — about 65% of the population. After this the number of eyed larvae declined, although the proportion of the total population which was eyed increased a little.

On any day the percentage of eyed larvae was roughly proportional to the mean size of the population, but as the larvae grew older the proportion eyed at a given mean size increased (Figure 13, 1963 and 1964 experiments). For example, on day 10 populations with a mean size between 280 and 290μ had 0 to 26% of their larvae eyed. On day 12 the range for populations of this size was 0–61%, on day 14 it was 15–75%, and on day 16 it was 24–79%.

This suggests that two factors were involved, one related to the size of the larvae and the other to the length of time that the larvae had been free-swimming. A population which had grown rapidly to a large size did not have such a large proportion of its larvae eyed *at that size* as a slower growing population when it reached that size. A rather similar phenomenon has been noted in ascidians (Grave, 1935), where it was found that a certain amount of activity was required before metamorphosis occurred.

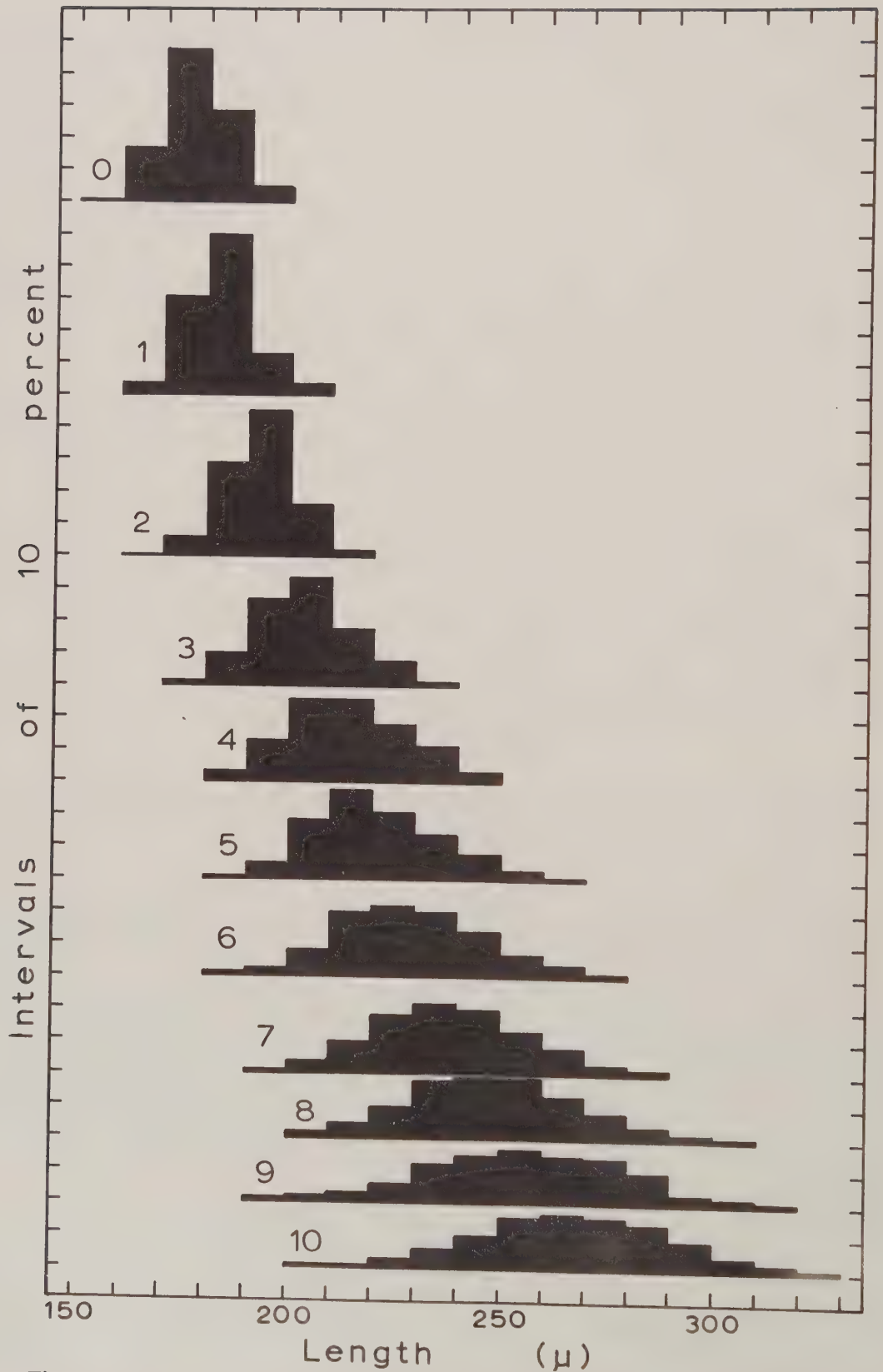

Fig. 9. *The mean size distribution of the larvae in the* 1963 *experiments on days* 0-10.

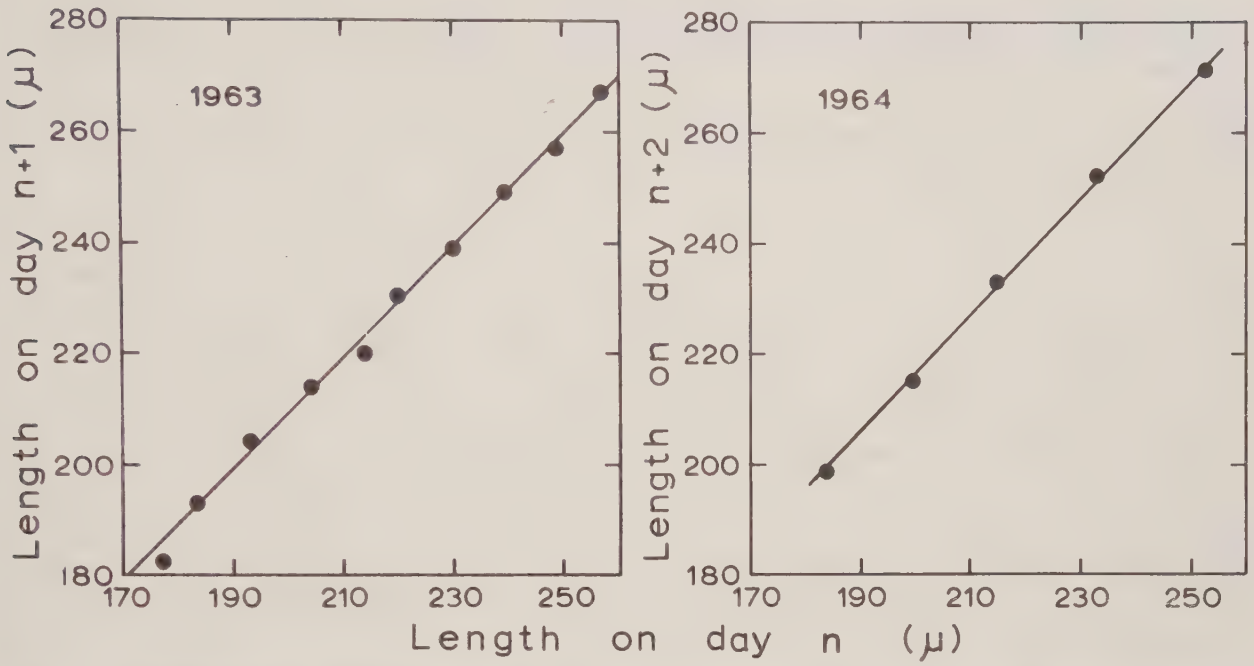

Fig. 10. *The mean length of the larvae set out in 'Ford-Walford' plots. For* 1963 *the length on day n is related to the length on day n+1; in* 1964 *the larvae were measured on alternate days, so the length on day n is related to the length on day n+2.*

In Figure 14 a series of histograms shows the percentage size frequency of the eyed larvae for successive days in the 1963 experiments. The majority were in the size range 280–309μ, but as the experiments progressed the mean size of the eyed larvae decreased (Figure 15, for 1963 and 1964 together). From these results it appears that a certain time must elapse before the larvae become eyed, and this results in the more rapidly growing larvae being larger when the eye develops than those which have grown more slowly. Cole (1939) has suggested that under various conditions growth in size and differentiation can proceed at different rates.

RELATION BETWEEN GROWTH RATE AND YIELD OF MATURE LARVAE

It was generally found that the more rapidly the larvae grew, the larger the numbers which reached a large size and subsequently become eyed. This is demonstrated in Figure 16, where a combined analysis of the 1963 and 1964 data relates (a) the mean length increment over the first 8 days and the proportion of the initial number of larvae reaching 270μ for individual experiments, (b) the mean length increment over the first 8 days and the proportion of the larvae becoming eyed, and (c) the proportion reaching 270μ and the proportion becoming eyed. Each of the three regressions is significant at the 1% level.

As the proportion of larvae which reaches 270μ or becomes eyed cannot be accurately calculated because some larvae will be continually growing into or leaving these categories, the following procedure was adopted. The records from individual experiments were examined, and from the size distribution data and estimates of larval numbers the maximum number of larvae 270μ or more in size on any one day was

determined. The same procedure was then adopted for the eyed larvae, and these figures were expressed as percentages of the number of larvae present when the experiments started. To smooth out irregularities in the estimate of the initial number, this has been calculated as the mean of the estimated populations on days 0 and 2.

Taking the average increment in the first 8 days as 70μ (Figure 8), then on the average 54% of the original larvae survived to grow to at least 270μ and 30% became eyed.

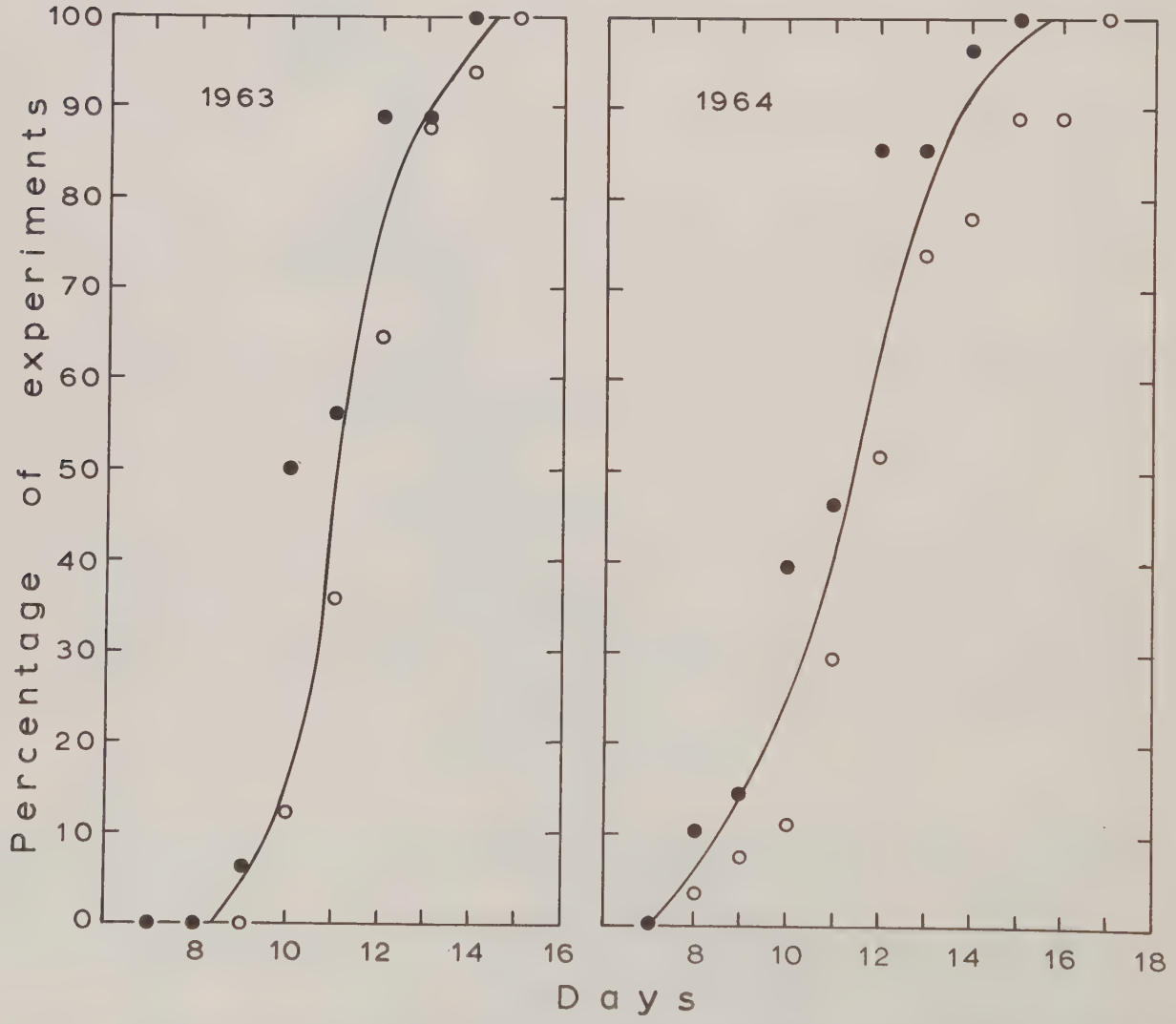

Fig. 11. *Cumulative curves showing the percentage of experiments with eyed larvae (solid circles) and the percentage in which spatfall was occurring (open circles).*

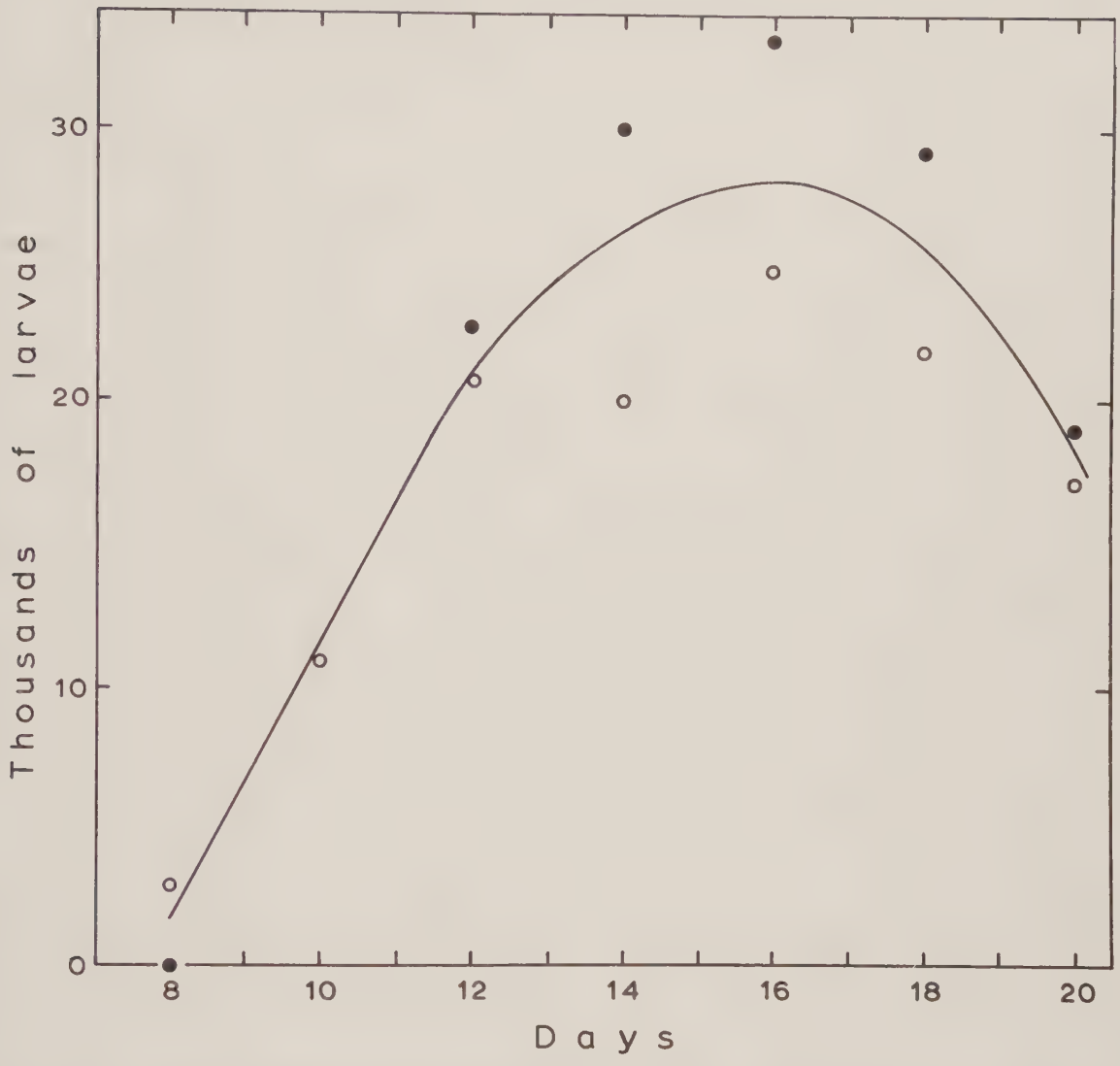

Fig. 12. *The average numbers of eyed larvae found at successive water changes in* 1963 (*solid circles*) *and* 1964 (*open circles*).

SPATFALL

This was recorded daily by counting the spat settled on two strings of five mussel shells. There was considerable variation in the density of spatfall in different experiments, but the skew curve given by the average results (Figure 17) is similar to that previously found in laboratory experiments (Bruce, Knight & Parke, 1940; Walne, 1956). Occasional spat were found in some experiments on days 10 and 11, and settlement usually reached a peak on days 14 to 16. After day 16 the number of experiments in which spatfall was occurring started to decline. It is noteworthy that although after 20 days the intensity of spatfall was low, some experiments still had substantial numbers of eyed larvae (Figure 12). Apparently eyed larvae which grow so slowly are not sufficiently vigorous to metamorphose, and a substantial spatfall is obtained only when the larvae grow rapidly.

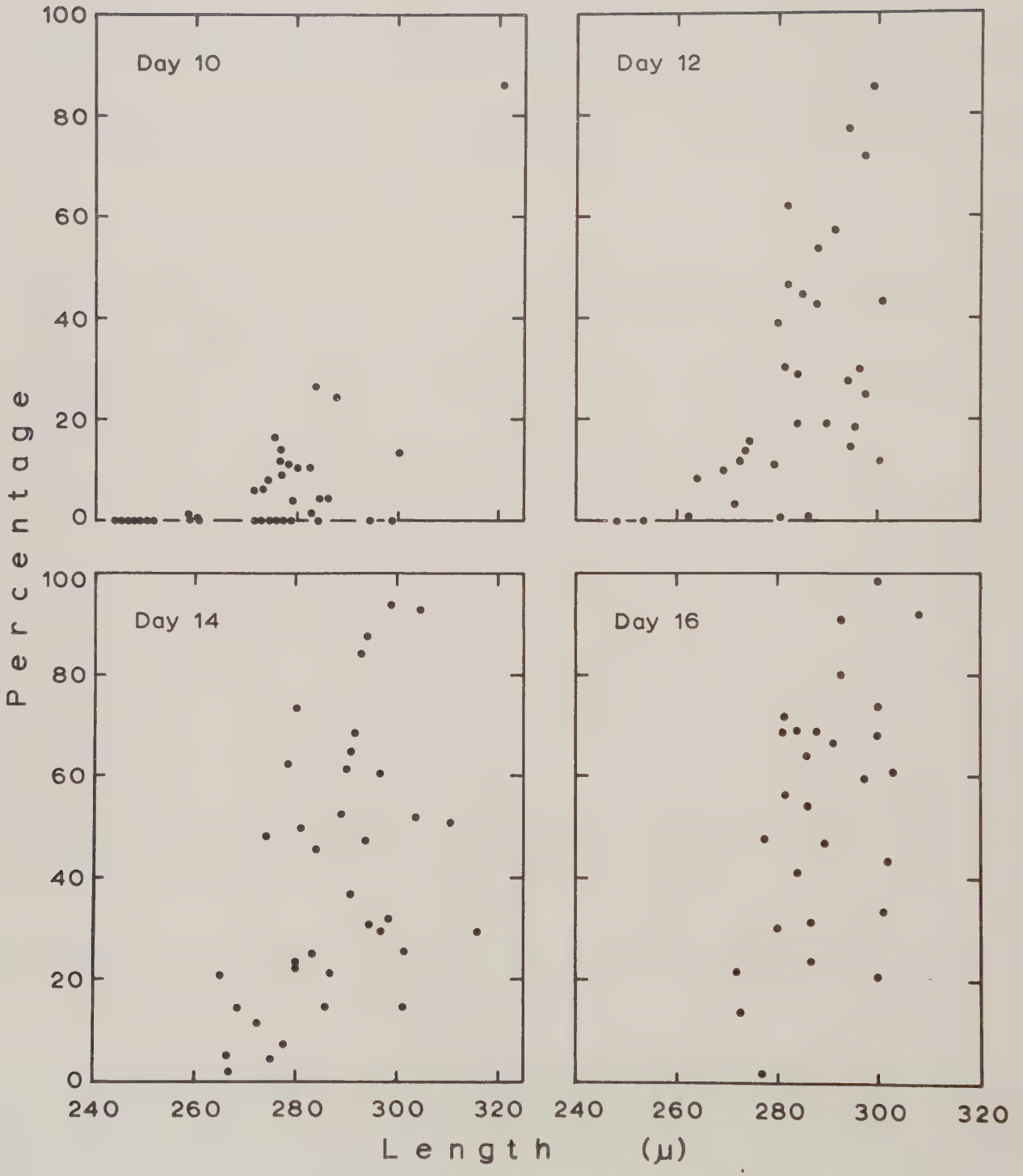

Fig. 13. *The percentage of eyed larvae related to the mean shell length in individual experiments on days* 10, 12, 14 *and* 16.

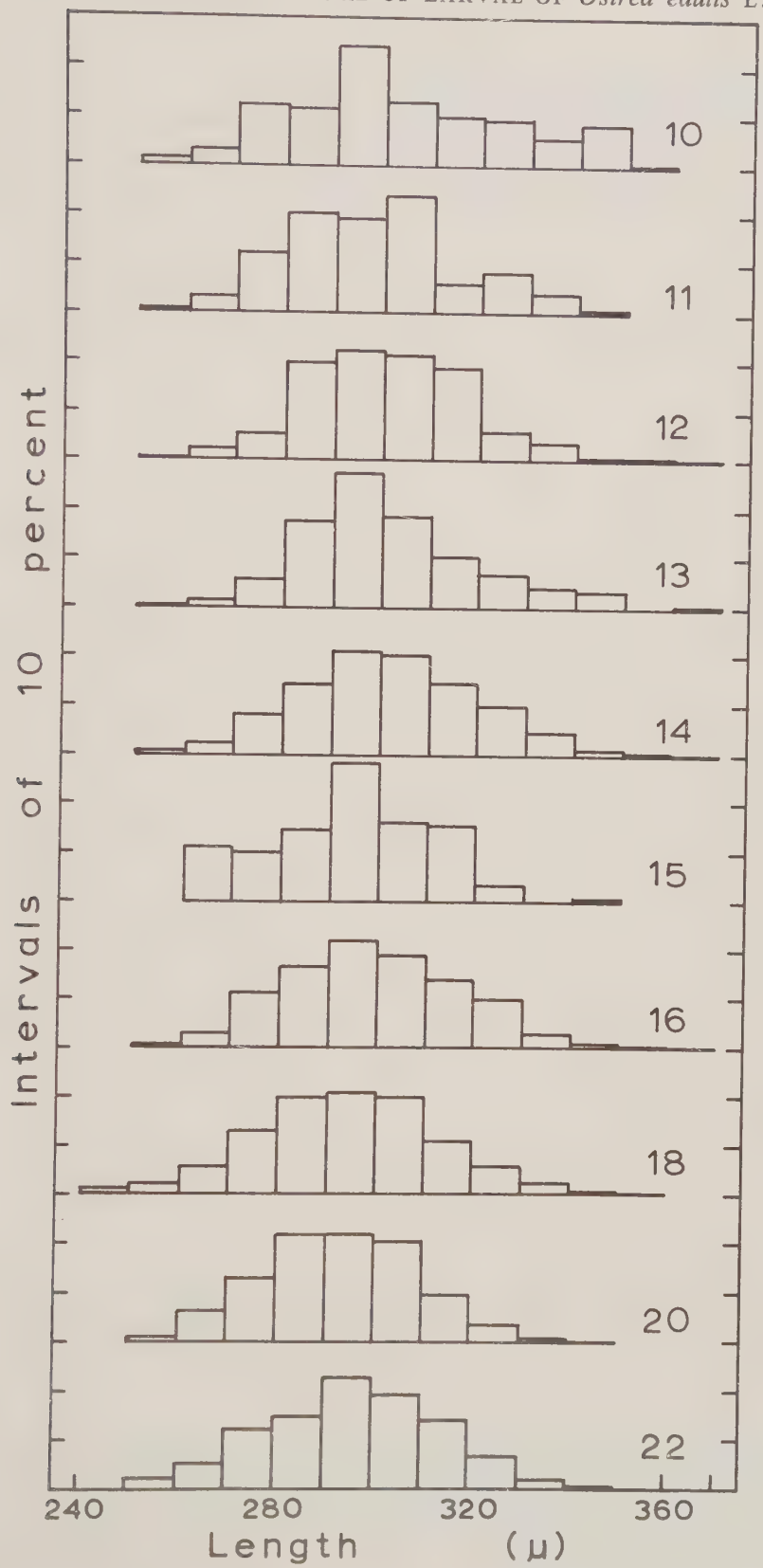

Fig. 14. *The average size distribution of eyed larvae on successive days in* 1963. *Equal weight has been given to each experiment in which eyed larvae were present.*

Although the shells gave a good indication of the duration of spatfall, the intensity of the spatfall on them was not closely related to that on the collectors as a whole. In some experiments the larvae tended to settle near the top of the bin, in others near the bottom, in some on under surfaces, in others on the upper surfaces of the collectors. The factors governing the behaviour of settling larvae under these conditions require further study; some experiments reported on p.48 suggest that light is important.

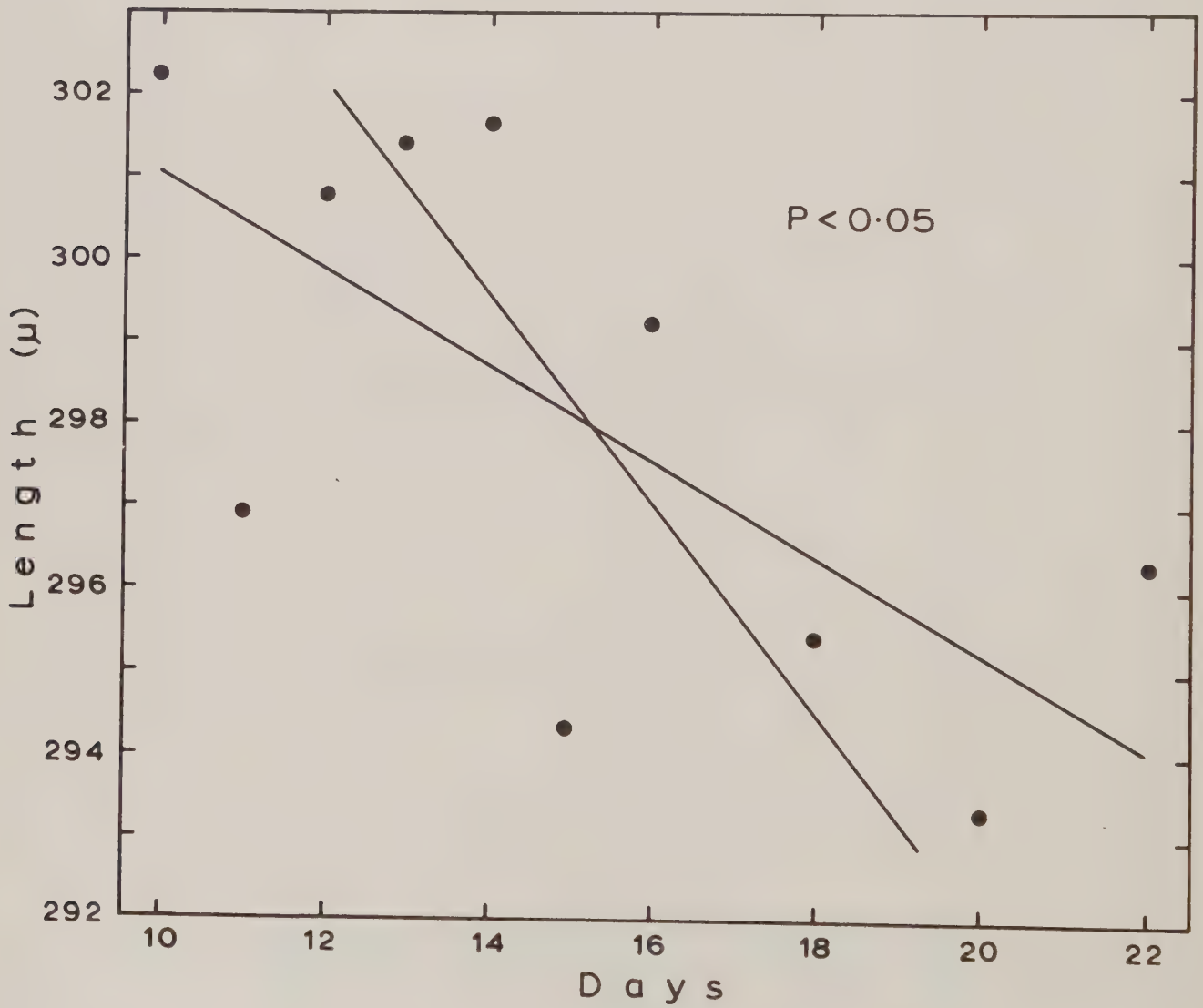

Fig. 15. *The mean size of the eyed larvae on successive days, calculated from the combined* 1963 *and* 1964 *data. The calculated regressions are shown.*

Plastic collectors were not usually added until eyed larvae had been present for a day or two; on the average they were first added after 11·6 days, with a range of 10 to 14 days (10-16 in 1964). In the earlier experiments only one bundle of eight collectors was added at a time, but this was found insufficient for the spatfalls obtained and the size of the bundles was increased to 16. In some experiments the collector had to be replaced after a few days. The average number of collectors added to an experiment was 20.4 with a range of 8 to 44. Spatfall tended to be more intense on one side of the collector than the other and the bundles were frequently turned over. Collectors which had been used before or had been well soaked appeared to be more attractive to settling larvae than new ones, and this factor may explain some of the variations of yield obtained. The importance of using collectors which had some degree of fouling has been discussed by Cole & Knight-Jones (1949).

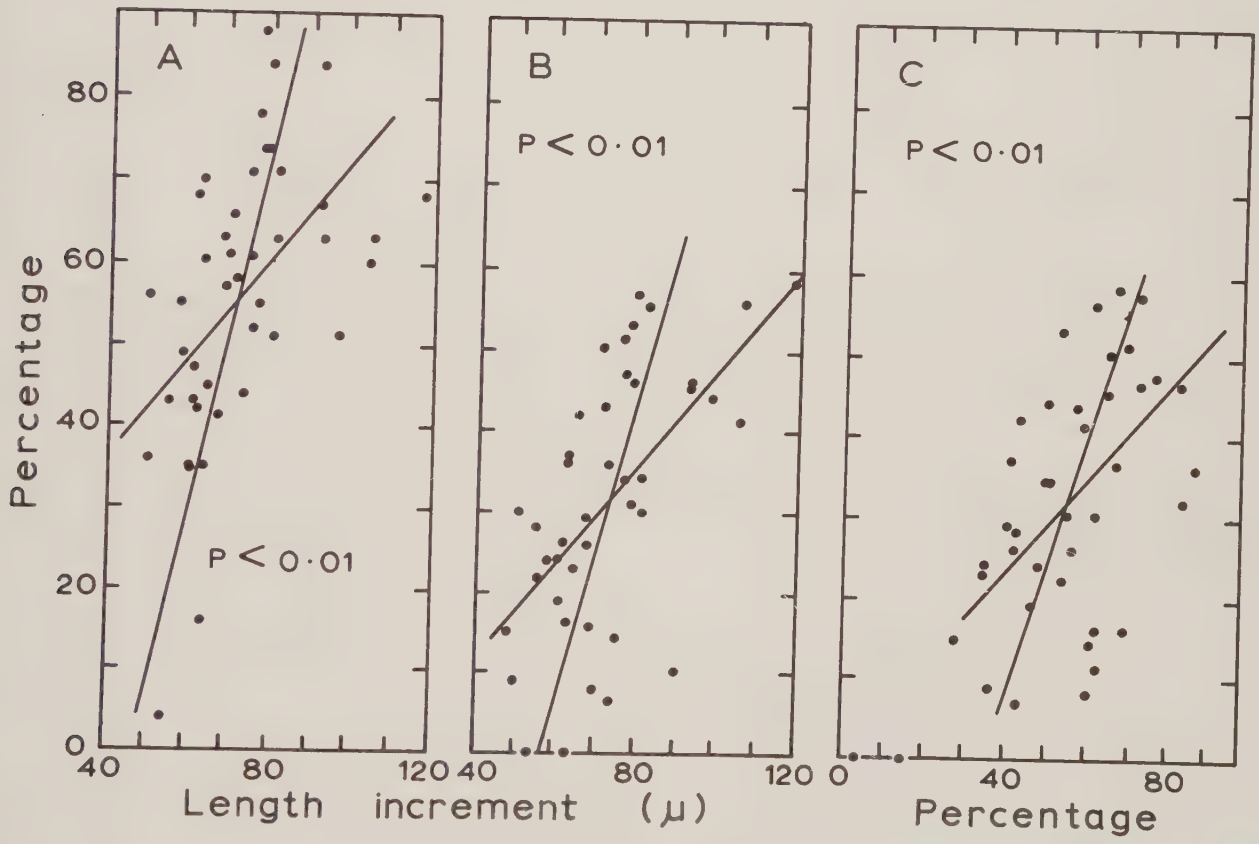

Fig. 16. *The relation between the growth rate and the proportion of larvae reaching* 270μ *and becoming eyed. The calculated regressions are shown.* (*A*) *The length increment in the first eight days after liberation, compared with the proportion of the original larvae reaching* 270μ; (*B*) *the length increment in the first eight days after liberation, compared with the proportion reaching the eyed stage;* (*C*) *the proportion reaching* 270μ (*abscissa*) *compared with the proportion reaching the eyed stage* (*ordinate*).

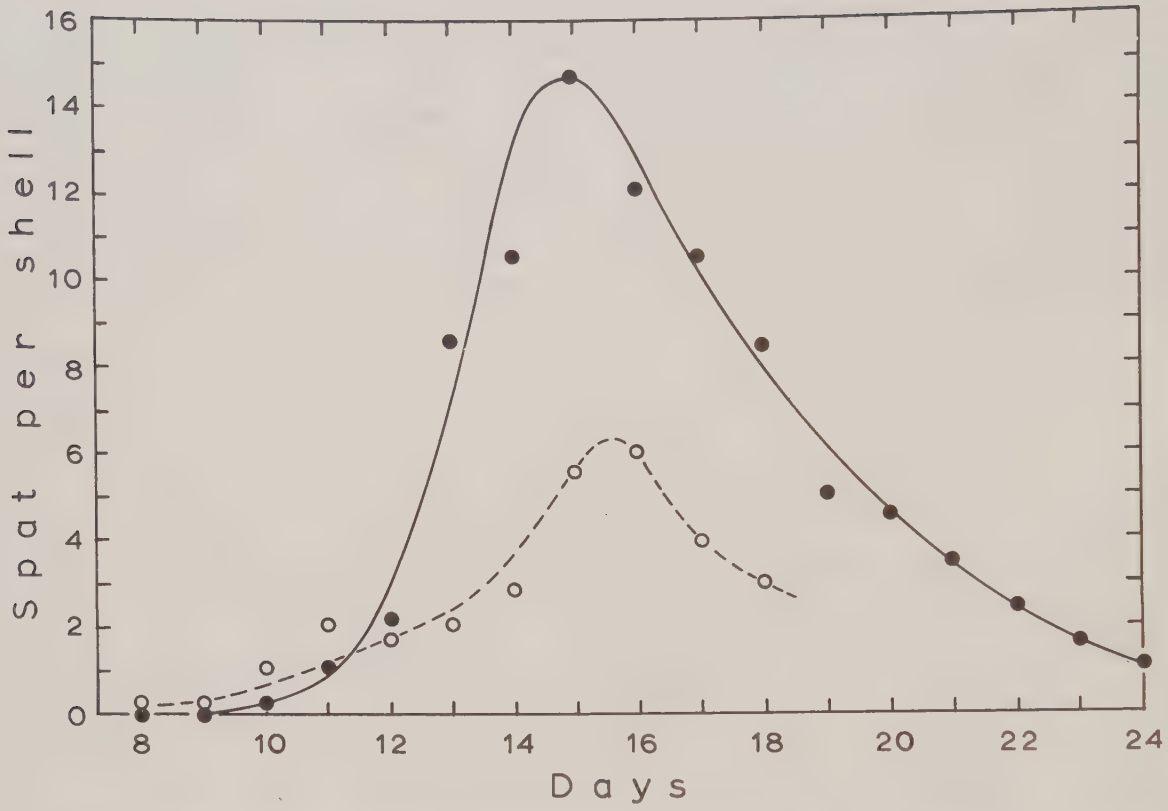

Fig. 17. *The average daily spatfalls on mussel shells in* 1963 (*solid circles*) *and* 1964 (*open circles*). *The data for* 1964 *after day* 18 *are omitted because two experiments had abnormally late and heavy spatfalls and their contribution to the average outweighed the data from the other experiments.*

Nineteen experiments were carried to completion in 1963 and 32 in 1964. The proportions which yielded spat in the two years were as follows:

	1963	1964
No spat	10·5%	25·0%
Spat on test shells but few on collectors	0	15·6%
Reasonable spatfall on collectors	89·5%	59·4%

Seven of the eight failures in 1964 were consecutive experiments started in the three-week period July 16 to August 5; possible explanations are discussed in more detail on p.37.

FOOD SUPPLY

In the 1963 season a detailed record was kept of the density of food, and the remarks in the following paragraphs apply in particular to that year. In the first part of the 1964 season an attempt was made to avoid the daily cell count by assuming that the cell density had fallen to the mean value given for that day

in the 1963 results. This was only partially successful, due to the considerable variation in feeding rate by different batches of larvae, and for the rest of the season the cell density was determined on the days when the water was not changed. In the latter half of 1964 the initial cell density was reduced from 120 to 100 cells per µl, and when the mean size of the larvae reached about 260 µ the food density was reduced to 75 cells per µl. Table 8 shows the mean cell density remaining on each day, and the concentration of *Isochrysis* on which the larvae had been feeding lay between this figure and the density to which the food was restored each day.

Table 8. *The average number of cells of* Isochrysis *left at the end of each 24-hour period in the larval rearing experiments*

Day	Number of cells remaining per µl	
	1963	1964
1	48	86
2	41	88
3	64	59
4	60	78
5	58	74
6	56	86
7	75	65
8	68	69
9	72	63
10	68	58
11	64	63
12	62	70
13	68	52
14	73	46
15	79	54
16	51	44
17	52	46
18	47	44
19	34	47

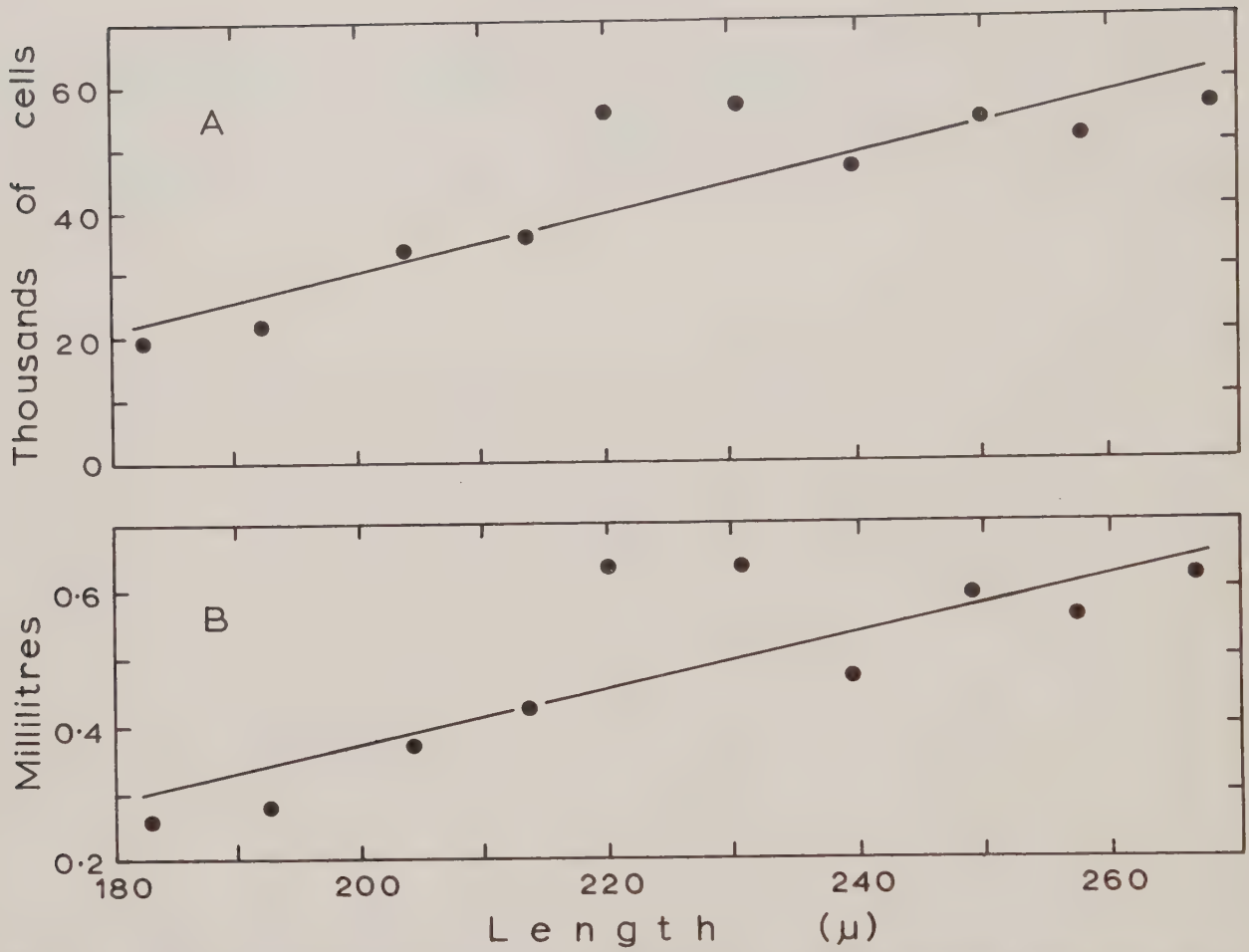

Fig. 18. (*A*) *The average numbers of* Isochrysis *cells eaten.* (*B*) *The average volume of water swept clear in* 24 *hours, related to the mean size of the larvae at the end of the period. Both figures are based on the* 1963 *data; the calculated regressions are shown.*

These data for the first ten days in 1963 have been used to calculate the average volume swept free (F) by a larva each day:

$$F = V.G.t,$$

where V is the volume of medium per animal, obtained from the data given in Figure 6, t is unit time (1 day), and G is the grazing rate defined by:

$$G = R - \frac{l}{t} \log_e \frac{P_1}{P_0} \quad ,$$

where P_0 is the number of cells per unit volume at the beginning of the experiment (120 per μl), P_1 is the number present at the end of 24 hours (Table 8), and R is the reproductive rate of the food (Jørgensen, 1943; Cushing, 1958). In this series of experiments R was not determined, because it had been found in other investigations to be negligible in the dark. The number of cells eaten is given by:

$$\frac{P_0 G}{R-G} \left(e^{(R-G)t} - 1\right).$$

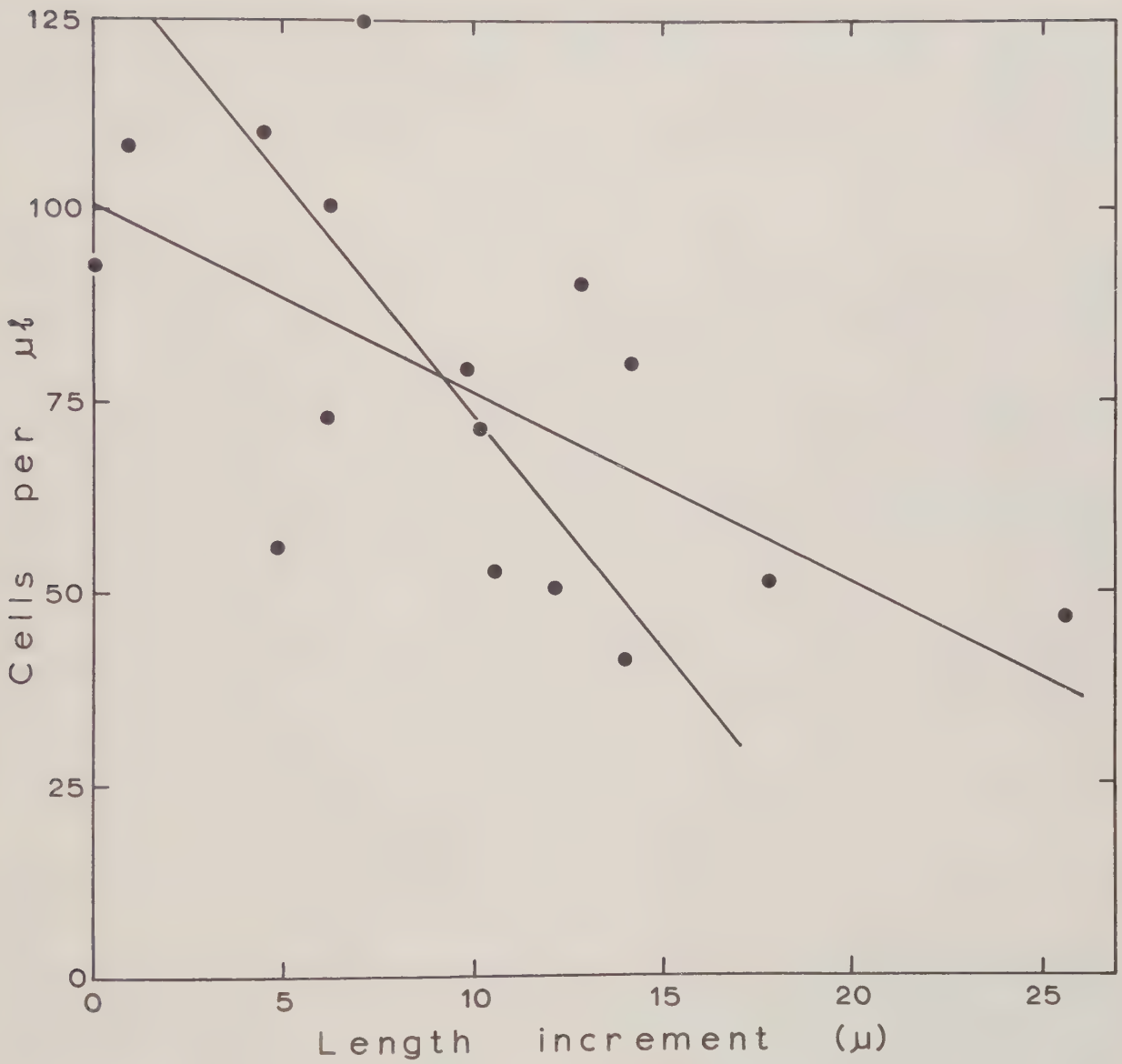

Fig. 19. *The number of* Isochrysis *cells left on day 7, related to the mean length increment of the larvae from day 6 to day 7 (from the data for the 1963 experiments).*

The regression of these measures of the feeding rate on the mean size of the larvae at the end of each 24-hour period (Figure 18) shows that the average volume swept free and the number of cells eaten per day increased by about three times during the first ten days.

The increased rate of feeding as the larvae grew larger was compensated for by the increasing volume available to the larvae (Figure 6); as a result, in 1963 the mean density of the flagellates tended to rise as the experiment proceeded until about day 16 when a sharp drop in numbers occurred (Table 8). This is thought to have been due to the presence at this stage of numerous metamorphosed spat, which apparently have a sharply increased grazing rate. Because of the greater volume, more food was available for the larvae in 1964.

During the first few days of an experiment no relationship was found between the larval growth rate and the number of flagellates left, but as the larvae grew larger a negative correlation occured. An example is given in Figure 19, where the length increment obtained between day 6 and day 7 is related to the number of *Isochrysis* cells left on day 7. It is clear that vigorous feeding and rapid growth were directly related and this suggests that food supply was not usually a limiting factor in these experiments.

BACTERIAL FLORA

The density of bacteria in the larval cultures was regularly estimated in 1963 by plating samples when the water was changed. These would represent the maximum densities to which the larvae were exposed.

The results obtained showed populations varying from a few thousand to over a million per ml. Some of this variability may have been due to experimental error, resulting in part from the difficulty of counting marine bacteria by the poured plate method because of their sensitivity to heat (Gunkel et al., 1961), but also from the dependence on a 1-ml sample which might or might not contain particles of debris which on disintegration would liberate many bacteria. At the higher counts the colonies were very dense on the plates and if further dilutions had been used the estimated densities would probably have been higher.

The average results (Table 9) suggest that there was an increase in the general level of bacteria during the first four days, which was reduced on days 5 and 6 by the daily water change and washing the larvae with chlorine solution. There was some increase again on days 8 and 10 but not to the level found on day 4.

Table 9. *The mean concentration of bacteria in the larval culture vessels at the time of the water change (1963 experiments)*

Day	2	4	5	6	8	10
Concentration (millions per ml)	0·69	1·42	0·44	0·46	0·57	0·88

No relation could be found between the growth of the larvae in a 48-hour period and the density of bacteria at the end of that period, but a consideration of the average level of bacteria in an experiment in relation to the larval growth rate suggests that where the mean level was high, the growth rate was reduced. In Figure 20 the length increment obtained in the first ten days of each of the 1963 experiments is related to the mean of all the bacterial counts made in that period (usually 6 counts, i.e. on days 2, 4, 5, 6, 8 and 10). The results are too variable for the apparent trend to be statistically significant, and it should be remembered that the method of observation would not reveal any variation in the composition of the bacterial flora which may have occurred.

PHYSICAL CONDITIONS

The range of salinities obtained from the seawater supply system during the two larval rearing seasons is given in Figure 1; it was usually between 31 and 34 $^{o}/_{oo}$.

Thirty-five estimates, spread over seven of the 1963 experiments, were made of the concentration of dissolved oxygen immediately before changing the water. A meter using a dropping mercury electrode was used, because the presence of antibiotics interferes with a Winkler titration. All but two of the estimates were in the range 95–105% saturation; the exceptions were in the range 90–95% saturation.

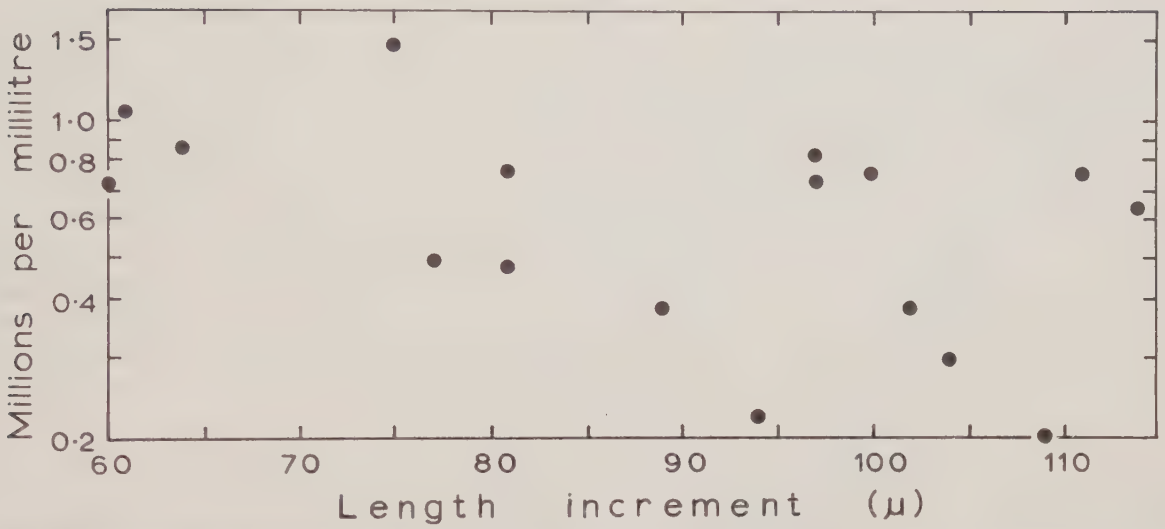

Fig. 20. *The growth increment of the larvae in the first ten days, related to the mean bacterial population at the times of the water changes during that period (from the data for the* 1963 *experiments).*

Temperatures were recorded daily in most experiments and were nearly always in the range 22·0–22·9°C. When the larvae were first put into clean water, the temperature was sometimes a few degrees below that being maintained in the hatchery. This was particularly the case in the early part of the season, because the heating system proved inadequate when relatively large volumes were suddenly drawn off to change the water in the experiments. It did not seem to affect the larvae and the water reached the full temperature in a few hours.

The pH was not observed but records obtained in earlier years, using either a pH meter or indicators, had shown that it was very constant at about 8·1.

TREATMENT OF THE SPAT

The following description is largely based on the system used in 1964. Briefly, the collectors were first placed in tanks of warmed and filtered sea water enriched with *Isochrysis* for a period after their removal from the experimental vessels, and from these they were moved to an outside tank of running sea water at ambient temperature. When the spat had grown to 8–10 mm they were stripped from the collectors and kept for a period in trays in running sea water until finally planted at the Ministry's Tal-y-foel oysterage. The time spent in each treatment by the larvae and spat in each experiment yielding a worthwhile number of spat in 1964 is given in Figure 21.

When the collectors were removed from the experimental vessels they were placed in 400-litre fibreglass tanks standing in the same room. These tanks were filled with sea water which had been filtered and sterilized in the same manner as that used for larval rearing, with the exception that antibiotics were not added. Two tanks were used, one above the other, and the water ran continuously from the top to the lower tank. A pump, controlled by a float switch in the top tank, returned the water and maintained a circulation. The temperature was kept at about 23°C. Cultures of *Isochrysis galbana* were added to the water as food; the cell density was normally restored to 75 cells per μl each day, and at times when a large number of spat were present a dosing pump continuously adding *Isochrysis* culture was used.

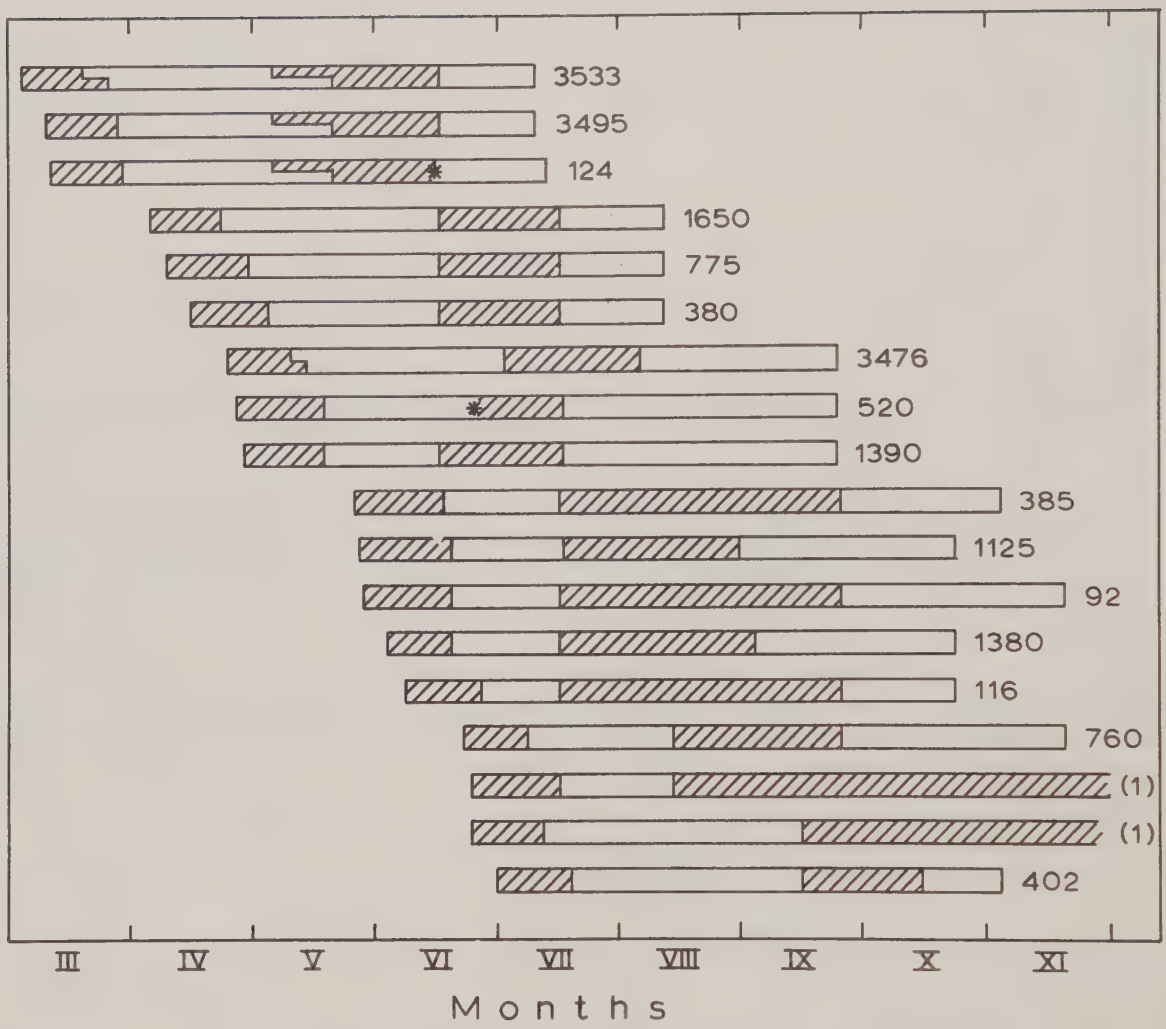

Fig. 21. *The period spent by each batch in the* 1964 *series of experiments successively as larvae, as spat in the hatchery, as spat on collectors in running water outside, and as spat stripped off collectors in running water outside. The number of spat when each batch was planted at the oysterage is shown.*
(1) *Remained on the collectors during the winter.*
* *Date not recorded.*

The water was changed and the tanks cleaned out twice a week. During the change the collectors were stood in fresh water for 5–10 minutes; this did not harm the spat, but helped to kill ciliates and other protozoa on the surface of the collectors. When the spat had grown to 2–3 mm in size, the collectors were hung in single file in an outside tank 38 cm wide by 48 cm deep by 3·7 m long. Water was siphoned from a 30 000-gallon storage tank, at about 7 litres per minute, into the bottom at one end of this tank and overflowed from the top at the other. When the spat had grown to about 1–2 cm, they were removed from the collectors. The bundle was taken apart and many spat would come off when the plastic sheets were sharply flexed; the remainder were taken off by scraping or brushing. Some shell damage was caused during removal, and while it was being made good by shell deposition the spat were kept in trays with a mesh bottom through which a flow of water was maintained. The number of live spat was then estimated by counting weigned sub-samples and they were removed to trays standing at low-water mark at the Tal-y-foel oysterage in the Menai Straits.

The duration of the various stages is shown in Figure 21. In general, the spat were kept in inside tanks for 3–6 weeks and then in sea water outside for 3–7 weeks. The period between stripping and planting at the oysterage was usually quite short, 2–5 weeks. Of course, those parts of the operation which depended upon ambient temperatures varied in length according to the time of year. In the early part of the season the spat were retained inside for a longer period until the water warmed up outside, whereas at the end of the season some spat had to overwinter on the collectors.

Eight experiments were completed in 1963 and 16 in 1964 to the stage of removing the spat to the oyster ground in the same season as it settled. The mean yields were 1 594 and 1 361 per experiment respectively. Spat from nine experiments were kept on the collectors in tanks at Conway for the 1963–64 winter, and the mean yield from these when they were finally planted during the following summer was 524. The collectors used are too flimsy to put out in the sea and Conway is not well situated for keeping spat overwinter.

SMALL-SCALE EXPERIMENTS, OTHER RELATED WORK AND DISCUSSION

In this section some aspects of the culture of larvae are discussed in more detail, data are presented from other experiments, and some of the possible lines of future developments are indicated.

FOOD DENSITY AND GROWTH

Small-scale experiments have suggested (Walne, 1963, 1965) that the maximum uptake of food by oyster larvae fed with *Isochrysis galbana* is obtained at a cell concentration of about 100 per µl, and that there is only a small reduction in uptake and growth if the cell density falls to about 50 per µl. For this reason an initial cell density of 120 cells per µl was selected for the 1963 experiments, since it was judged that, at the larval densities used, about half of the food would be eaten overnight. The larvae would therefore be feeding in the range of 60–120 cells per µl and their growth would be largely unaffected by variation in the food density. As shown earlier this was not quite achieved in the first 48 hours of the 1963 experiments, because the density of the larvae was too great and it is probable that competition for food affected growth. This was overcome in 1964 by doubling the volume of water in the first 48 hours.

In 1964 a series of growth experiments was made at cell densities of 120, 90, 60 and 30 per µl in 1-litre beakers. Samples of about 200 larvae, collected at 48-hour intervals from two different broods, were added to each beaker and grown for 48 hours. The results (Table 10) show that the average length increment obtained was similar throughout the range of food densities tested, and neither the higher nor lower food densities had an adverse effect on the growth rate. Previous experiments (Walne, 1965) had suggested that cell densities as low as 30 per µl would have an adverse effect on growth. It may be that the present larvae were in better condition than those used in the previously reported experiments, and that part of the growth increment of those in the sparser food was a reflection of their previous history. Shell growth can continue for one or two days in the absence of food. This is demonstrated by the following data, which give the average length of a batch of larvae kept for six days after liberation in filtered sea water:

Day 0	182·6µ
,, 2	190·6µ
,, 4	190·0µ
,, 6	192·6µ

To keep larvae at densities greater than 1 000–1 500 per litre it is necessary to add food more frequently than every 24 hours, since otherwise the rate of grazing reduces the food to a very low level. A number of experiments of this type were made in 1961. The larvae were cultured in fibreglass tanks holding 100 litres of filtered sea water to which antibiotics had been added; the water was kept stirred by a perforated

Table 10. *The length increment of larvae in 48 hours, when cultured at* Isochrysis *densities of 30, 60, 90 and 120 cells per μl. All the larvae came from one or other of two broods of larvae (1964 experiments)*

Brood of larvae	Initial size (μ)	Mean increment (μ) at:—			
		30 cells/μl	60 cells/μl	90 cells/μl	120 cells/μl
2	219·8	23·5	23·4	28·3	19·0
2	230·0	18·9	14·8	17·4	13·3
1	245·3	19·3	18·5	22·1	16·5
2	247·9	22·0	21·3	25·7	25·3
1	260·6	26·4	26·2	24·6	25·0
2	263·8	17·6	14·2	18·0	17·4
1	287·5	16·9	16·0	15·5	13·7
1	298·4	4·1	7·3	8·7	13·2
Mean increment (μ)		18·6	17·7	20·0	17·9

plastic plunger plate. An apparatus based on a timing device and electro-magnetic valves dosed a weighed quantity of *Isochrysis* culture into the experiment every three hours, but it was not very reliable and frequently too little or too much culture was added. The growth rates of three experiments are detailed in Table 11; they compare favourably with those obtained in the 1963–64 experiments, but it was found in these and others that it was rarely possible to keep the larvae growing vigorously after eight days. With this density there was a considerable accumulation of faecal debris in 24 hours and this became progressively worse as the experiment proceeded. Increasing the volume available to the larvae as they grew larger, or changing the water more frequently, would overcome this difficulty. These experiments are however sufficient to show that it is possible to rear larvae for at least part of the time at much higher densities than used in the main experiments detailed in this paper. The development of micro-dosing pumps of various types in recent years, which would allow the food supply to be accurately metered, suggests that this line might profitably be explored further.

CONTAINERS

There is some evidence that the shape and composition of the containers used for larval culture are significant factors. The largest glass containers readily obtainable were moulded tanks holding 45 litres. For the first experiments in larger volumes fibreglass tanks 22 × 32 × 10 inches deep holding 100 litres were used. When these were agitated by plunger plates it was observed that there was a tendency for the larvae to settle out in the corners of the tanks. Following the observation that plunger plates reduced survival (see p.45), several experiments were made in which stirring was provided by up to 12 air jets (see p. 46). These were difficult to manipulate and not entirely successful. Observations reported in the literature that many planktonic forms are stimulated to swim by pressure changes (Knight-Jones & Qasim, 1955; Bayne, 1963) suggested that a greater depth of water might be better. Some preliminary experiments were made in which batches of larvae from large-scale experiments were kept for 48 hours in vertical hard-glass tubes of $\frac{5}{8}$-inch bore. Two 36-inch lengths were connected together through a stop-cock, and by closing this the numbers in the upper and lower halves could be estimated. The tubes were

kept in the dark and the total volume was only 320 ml. In a sample of 3 000 larvae from one batch with a mean length of 228μ, 95% were in the top half in six hours. Samples from the same batch of larvae were tested over three consecutive 24-hour periods as their mean size increased from 204 to 215μ, and in each case over 95% were in the top tube. Samples from two other batches were examined on each of six consecutive days, and although these were on the average more equally distributed it was noticed that, on ten out of the eleven occasions when the mean sizes of the larvae in the top and bottom tubes were determined, more of the larger larvae were in the top tube. The larvae were at a density of a thousand or more per litre, and conditions in such a narrow tube cannot have been ideal, but the results suggest a tendency for healthy larvae to keep in the upper part of a long water column. It is noticeable that in healthy undisturbed cultures numerous larvae will 'raft' against the surface film. The polythene bins used for the main series of experiments detailed in this paper had the advantage of providing a taller water column than the fibreglass tanks, and a single central aerator provided a current system similar to that found in a 1-litre beaker. This did not leave the 'dead' areas characteristic of rectangular tanks.

Table 11. *Three experiments made in 1961, showing the mean size and percentage survival of high densities of larvae grown in 100 litres in fibreglass tanks. The cultures were stirred by agitation, and additional food was added automatically every three hours*

Day	Initial number of larvae					
	750 000		1 060 000		1 013 000	
	Size (μ)	%	Size (μ)	%	Size (μ)	%
0	182	100	183	100	183	100
2	203	88	205	82	202	94
4	242	–	–	–	231	65
5	–	–	251	77	–	–
6	271	48	–	–	247	43
7	–	–	267	75	–	–
8	284	44	–	–	259	21

There is some suspicion that polythene vessels are, at times, less favourable for larval growth than those of glass. During the period in 1964 when larvae were difficult to rear (see p.00) some other experiments were being made in glass beakers and these were set up by dipping 1-litre samples out of the 75-litre polythene bins immediately after the water change. The conditions were therefore identical except for the total volume and the composition of the vessel. It was found in these experiments that the larvae grew much better in the beakers than in the polythene bins. This difference was then examined in more detail in the following experiment. A standard large-scale experiment, which had been in progress for four days, was changed in the normal way and then thoroughly agitated before four 1-litre samples were dipped out. Two of the samples were cultured in hard-glass beakers and two in polythene vessels of about the same size. One of each pair stood on a bench in the hatchery and one in a water bath in a laboratory. Both in the 75-litre and the 1-litre experiments the water was changed as usual every 48 hours.

These experiments were therefore identical as regards water, food and larvae, but as the results show (Table 12) those in glass grew more than those in polythene. Those kept in the hatchery grew a little faster than those in the laboratory; the latter were about 2°C cooler. It is interesting that the larvae in the 75-litre polythene bins grew better than those in the 1-litre polythene vessels, which may have been a reflection of the relatively reduced surface area of polythene in contact with the water. Although these data suggest that polythene can be unfavourable, compared with glass, nevertheless many very good results have been obtained in polythene vessels. As a preliminary hypothesis it is suggested that with certain water conditions, perhaps when there is an abnormally high content of dissolved organic matter, then polythene vessels become markedly unfavourable, compared with glass, because of their ability to harbour a larger bacterial flora on their surfaces. Materials suitable for the construction of large culture vessels need further study and comparison with glass.

Table 12. *The mean length of larvae with an initial size of 233μ compared in 1-litre glass and polythene beakers and in a standard 75-litre polythene bin. One of each pair stood in a laboratory and one in the hatchery. Larval density about 1 100 per litre*

Day	Mean length of larvae (μ) in				
	1-litre glass beakers		1-litre polythene beakers		75-litre polythene bin
	In laboratory	In hatchery	In laboratory	In hatchery	In hatchery
2	257	260	235	241	254
4	270	285	237	246	263
6	275	293	dead	249	274

BACTERIA AND ANTIBIOTICS

Earlier work (Walne, 1958) had demonstrated that an increased yield of spat is obtained if the larvae are cultured in the presence of 50 000 i.u./l penicillin G and 50 mg/l streptomycin sulphate, and this dosage has been used in the experiments reported in this paper. Further experiments with a few hundred larvae in 1-litre beakers have shown that the effect of antibiotics could occasionally be detected in the growth obtained in as little as four days (Table 13).

Table 13. *The mean length of larvae grown in paired 1-litre hard-glass beakers and fed with 100 cells* Isochrysis *per* µl *(results of duplicate sets of measurements). The standard dose of antibiotics was 50 000 i.u./litre of penicillin G and 50 mg/litre of streptomycin sulphate*

Initial size (µ)	*Days from commencement of experiment*	Mean length (µ)	
		Without antibiotics	*With standard dose of penicillin and streptomycin*
190	2	225 and 226	228 and 230
	4	255 and 251	255 and 258
	6	276 and 279	287 and 287
	8*	286 and 287	296 and 317
175	2	205 and 200	198 and 201
	4	232 and 222	234 and 231
	6	251 and 249	253 and 255
	10	307 and 310	303 and 319
214	2	234 and 233	229 and 232
	4	241 and 248	250 and 251
	* % eyed on day 8	23 and 20	33 and 59

In Table 13 are reported the results of three series of experiments in which the larvae were fed on *Isochrysis* at about 100 cells/µl and the water was changed every 48 hours. In each batch, two beakers were cultured in the presence of antibiotics and two controls were grown without. Although the differences in mean size were small, on every day but one the average size of the larvae was greater in those experiments in which antibiotics were present. In the first series the proportion of eyed larvae present at the end of the experiment was much higher when grown in the presence of antibiotics. It has been generally found that the effect of antibiotics in increasing the growth rate becomes more marked as the larvae approach settling size. This is particularly noticeable when the results of similar but larger-scale experiments are considered (Table 14). In none of these was the growth rate substantially different during the first eight days, but from then on those which had been cultured in the presence of antibiotics grew ahead and by days 10–15 they were larger and with a much higher percentage alive than those without antibiotics. None of the latter produced eyed larvae or spat, while those with antibiotics produced both.

The earlier work (Walne, 1958) had shown that a penicillin-streptomycin mixture was more reliable than either antibiotic on its own, and an experiment with 25 mg/l of chloromycetin gave poor results. Four further series of experiments in the 1-litre beakers extended these observations. In the first series

Table 14. *The growth and survival of larvae in large-scale experiments when reared in the presence or absence of antibiotics (50 000 i.u./litre of penicillin and 50 mg/litre of streptomycin sulphate). Experiment 1: water changed every 48 hours. Experiments 2 and 3: water changed every 48 hours in bins with antibiotics, and every 24 hours in those without*

	Days from commencement of experiment	Without antibiotics	With standard dose of penicillin and streptomycin
Experiment 1 About 500 000 larvae cultured in a 100-litre fibreglass tank	0	175	175
	2	204	205
	4	232	236
	6	259	264
	8	272	289
	10	265	307
	% eyed	0	33
	% alive	14	64
Experiment 2 About 500 000 larvae cultured in 75-litre polythene bins	0	186	186
	2	210	210
	4	227	232
	6	240	244
	8	242	248
	10	242	261
	% alive	Majority dead	83
Experiment 3 About 300 000 larvae cultured in 75-litre polythene bins; most of those in the experiment without antibiotics were dead on day 15	0	182	182
	7	232	–
	8	–	231
	13	265	–
	14	–	273
	% eyed	0	1
	% alive	35	57

larvae initially measuring 193·4μ were grown for eight days in the presence of 12·5, 25·0 and 37·5 mg chloromycetin/litre. Each treatment was in duplicate, the water being changed every 48 hours, with *Isochrysis* added as food. The mean size of the larvae after eight days (shown as the results of duplicate sets of measurements) demonstrates the better growth obtained with the standard antibiotic mixture than with chloromycetin:

Antibiotics per litre	Mean length (μ)
50 000 i.u. penicillin, 50 mg streptomycin (Controls)	281 and 283 (after 6 days only)
12·5 mg chloromycetin	267 and 272 (after 8 days)
25·0 mg chloromycetin	269 and 259 (after 8 days)
37·5 mg chloromycetin	239 and 241 (after 8 days)

This suggests that increasing the dosage of chloromycetin decreases the rate of growth. In the next experiment larvae with an initial mean size of 190μ were cultured for eight days in the presence of a smaller dose of chloromycetin, but again the growth rate was inferior to that of the controls.

Antibiotics per litre	Mean length (μ) after 10 days	% eyed
50 000 i.u. penicillin, 50 mg streptomycin (Controls)	318 and 330	94 and 94
50 mg streptomycin	286 and 284	56 and 20
25 mg streptomycin	295 and 265	52 and 4
6 mg chloromycetin	272 and 283	6 and 0

This series also shows that although those cultures with only streptomycin did well, their growth was inferior to that obtained with the larvae cultured with the penicillin-streptomycin mixture.

Aureomycin was tested at three different concentrations on larvae with an initial length of 161·8μ, and on this occasion the control had no antibiotics. The mean lengths after 12 days were:

Antibiotics per litre	Mean length (μ) after 12 days
None (Controls)	256 and 241
2·2 mg aureomycin	226 and 224
3·4 mg aureomycin	224 and 242
5·6 mg aureomycin	233 and 224

These data suggest that these dosages are unfavourable to larval growth. In a further experiment lower dosages of aureomycin were tried, and in addition the effect of growing the larvae alternately in the usual penicillin-streptomycin mixture and in chloromycetin in consecutive 48-hour periods was examined. The larvae, initially 175μ in size, were cultured for 12 days and, as the following data demonstrate, the best results were again obtained in the standard penicillin-streptomycin mixture:

Antibiotics per litre	Mean length (μ) after 12 days
50 000 i.u. penicillin, 50 mg streptomycin	311 and 319
6 mg chloromycetin	293 and 302
Alternately penicillin/streptomycin and chloromycetin	307 and 301
Alternately chloromycetin and penicillin/streptomycin	297 and 296
0·28 mg aureomycin	260 and 264
0·56 mg aureomycin	253 and 276
1·12 mg aureomycin	276 and 275
None	307 and 308

Four series of experiments were made to investigate whether water in which larvae had been cultured was suitable for the further growth of larvae. In the first series water from a large-scale experiment in which larvae at 2 500 per litre had been cultured for 48 hours was filtered free from larvae and added to 1-litre glass beakers. In addition some of the same water was filtered through a cellulose and mineral fibre filter to remove accumulated bacteria. Freshly drawn and filtered sea water was used as a control. Sufficient *Isochrysis* was added to all vessels to give 100 cells/μl, and 50 000 i.u. penicillin and 50 mg streptomycin/litre were added to the control. About 200 larvae with a mean length of 181μ were added to each vessel. After 48 hours the mean lengths were:

Treatment	Mean length (μ)
Unused water (Control)	207 and 207
Water used by larvae for 48 hours (unfiltered)	200 and 203
Water used by larvae for 48 hours and then filtered	192 and 193

It is evident that the used water was not quite so favourable, and that filtering was harmful. The experiment was repeated using water from a culture containing 4 300 larvae per litre. The growth of larvae with an initial length of 203μ in 48 hours was as follows:

Treatment	Mean length (μ)
Unused water (Control)	222 and 222
Water used by larvae for 48 hours (unfiltered)	221 and 222
Water used by larvae for 48 hours and then filtered	217 and 213

On this occasion there was no difference in the growth obtained in used water compared with unused, but again filtering had a harmful effect. In a further series with larger larvae (252μ) the used water was harmful:

Treatment	Mean length (μ)
Unused water (Control)	269 and 272
Water used by larvae for 48 hours	266 and 261

In the final series the growth of large larvae (279μ) was observed in water which had been used for only 24 hours for culturing larvae at 1 660/litre. In addition to testing filtered and unfiltered water, the effect of adding standard antibiotics to used water was observed. The mean sizes of the larvae after 48 hours were:

Treatment	Mean length (μ)
Unused water (Control)	300 and 301
Water used by larvae for 24 hours	298 and 293
Water used by larvae for 24 hours and antibiotics then added	301 and 293
Water used by larvae for 24 hours and then filtered	288 and 284

From all these results it appears that generally water in which larvae have been cultured for 24–48 hours is less favourable than unused water for further larval growth, and its filtration through a cellulose-mineral fibre filter to remove the accumulated bacterial flora makes it even more unfavourable. This may be due to the adsorptive properties of the filter or to something leaching into the water. For satisfactory growth at these larval densities, it is best to renew the water regularly.

Assuming that bacteria are an important factor in the culture of larvae, it is therefore desirable to reduce the contamination at each water change to the lowest level and to control the ingress of air-borne bacteria. The importance of the latter was demonstrated when it was found that an uncovered beaker of autoclaved sea water would develop 1 800 bacteria per ml in 24 hours and 500 000 in 48 hours if stood on a bench in the hatchery. Aerial contamination was controlled when the vessels were covered. The sources of the initial bacterial flora are the larvae, the algal food and the water, and these are now discussed in turn.

It has been observed that when the larvae have been well washed and resuspended in 1–2 litres of freshly filtered water the suspension contains up to 20 000 bacteria per ml, and it is probable that many of these are attached to the larval shell. For this reason experiments were made to find out whether washing the larvae in a dilute chlorine solution would drastically reduce the number of bacteria carried over from one water change to the next. This could be particularly important if harmful strains tended to accumulate during an experiment. Tests were made in which larvae were washed for five minutes in about 3 ppm chlorine and then plated individually. After 48 hours' incubation such preparations generally showed no bacterial growth. Tests in which batches of larvae were stood for five minutes in 1 litre of 3 ppm chlorine and then washed into filtered sea water showed that the bacterial flora had been reduced to a few hundred per ml.

Very substantial variations in the bacterial flora of the *Isochrysis* cultures have been recorded, and when the culture is added at the rate of 10 ml per litre of sea water this results in additions of the order of 200-20,000 bacteria per ml. Some attempts were made to reduce the number of bacteria by centrifuging the culture and resuspending in sterile water, but it was found that the centrifuging also brought down most of the bacteria. Surprisingly it was also found that *Isochrysis* cells treated in this manner were less suitable than the unwashed *Isochrysis* for the growth of oyster larvae. Attempts were made to find out whether some components of the *Isochrysis* medium had a stimulating effect on larval growth, but none was found. It is possible that centrifuging and then resuspension of the cells broke some of them and that their subsequent decomposition was unfavourable. Experiments with bacteria-free cultures did not necessarily produce better growth (Walne, 1963).

Considerable difficulty has been found in treating the several hundred litres of sea water required each day so that it was delivered into the larval culture vessels with only a low bacterial contamination. During 1964 a weekly check was made of the bacterial flora in various parts of the system by sampling the warmed water as it entered the sand filter, after it left the ceramic filter, and finally as it entered the larval culture. A preliminary investigation showed that the ratio of the bacterial counts from the three sites was 1·0:5·8: 2·0 and this demonstrated that the technique used, combined with the routine change of the filter candles and flushing of the pipework with fresh water, was failing to keep the bacteria under control. After this a solution of 3 ppm of chlorine in fresh water was pumped through the system for five minutes every day before use, and the mean ratio of the routine counts for thirteen successive weeks then became 1·0:0·97: 0·26. Although this represented a substantial removal of bacteria, a population of 90-1,300 per ml remained in the larval cultures. As this is the same order of numbers as are present in the water when it comes from the sea, it shows that the filtering and the ultra-violet unit are only just keeping pace with the contamination from the various tanks and pipes through which the water comes. It is clear that to prepare this volume of water substantially free from bacteria it would be necessary to design a unit which could be frequently and thoroughly cleansed of all residual contamination.

STIRRING

In the experiments in 1-litre beakers, gentle stirring was provided by bubbling air at 0·5-1·0 litre/minute from a glass jet held near the bottom. Similar methods and rotary stirring were not suitable for rectangular tanks (see p.36). Perforated plastic plunger plates were tried in the 100-litre tanks and these were reasonably successful in keeping the larvae in suspension, but observation suggested that the plates were damaging the shells of the larvae. This was tested by adapting the apparatus to operate in 1-litre beakers. Four replicate beakers standing together in a water bath were set up with larvae of a mean size of 218μ and with *Isochrysis* added as food. Two of the beakers were agitated with plunger plates, while two were stirred with air bubbles. After 48 hours the larvae were measured; those that had been agitated had a mean size of 242μ, while those that had been stirred with air bubbles had a mean size of 254μ. In addition, 46% of the larvae in the agitated experiment had cracked shells, while only 3·5% of those which had been air-stirred were damaged.

A similar experiment in 1-litre beakers compared the value of stirring with air bubbles against stagnant cultures. Two pairs of beakers were stood in the normal laboratory light and two pairs in the dark. The beakers were each stocked with about 4 000 larvae of a mean size of 204μ. The enhanced vigour of the larvae in the stirred cultures is suggested by these estimates (from duplicate counts) of the number of *Isochrysis* cells remaining after 24 hours:

	Number of cells per µl	
	In the light	In the dark
Stagnant	73 and 72	63 and 66
Aerated	31 and 41	38 and 50

After 48 hours the larvae were measured and the following mean sizes obtained:

	Mean length (µ)	
	In the light	In the dark
Stagnant	207 and 213	212 and 210
Aerated	224 and 218	216 and 218

Evidently in these conditions stirring was beneficial, and it is thought that the benefit comes from the stimulation of the larvae due to the turbulence caused by the air bubbles, rather than from the direct effect of maintaining the oxygen content of the water.

An experiment lasting six days in two 40-litre glass tanks with 300 000 larvae in each provides further evidence that a fair degree of stirring by air bubbles is advantageous. One of the tanks was stirred by a single jet, while in the other a more vigorous turbulence was obtained by using nine jets. The larvae were put into the tanks on liberation, when they measured 178µ. Growth was similar for the first four days but on the fifth day the larvae in the tank with one jet began to settle out, and by the sixth day these larvae were markedly smaller (mean length 227µ) than those in the tank with nine jets (240µ). The larvae were then transferred to 100-litre fibreglass tanks, fitted with one jet and twelve jets respectively. The larvae which had been more vigorously stirred all along reached a mean length of 265µ by day 10, while the others were only 242µ at this time.

OXYGEN REQUIREMENTS

The following observations made on the oxygen consumption of oyster larvae suggest that, at the larval densities employed, only a small part of the oxygen content of the water is used. The oxygen consumption of oyster larvae at the temperatures prevailing in the large experiments were estimated in two series of experiments in 1963, by observing the removal of oxygen by fairly high concentrations of larvae when kept in stoppered bottles.

In the first series freshly liberated larvae with a mean length of 189µ were added at different densities to bottles holding about 300 ml of filtered sea water. Sufficient *Isochrysis* was added to give about 50 cells/µl. The oxygen content of the water was determined at the beginning of the experiment, and after about 21 hours, by an oxygen meter using a dropping mercury electrode. The number of larvae and the volume of the bottle were measured and from these data the oxygen consumed by 10 000 larvae in 24 hours has been calculated; in Figure 22 this is related to the density of larvae. The higher densities show a reduced consumption, and an extrapolation of the curve to lower densities of larvae suggests that 10 000 could consume at least 1 ml oxygen per day.

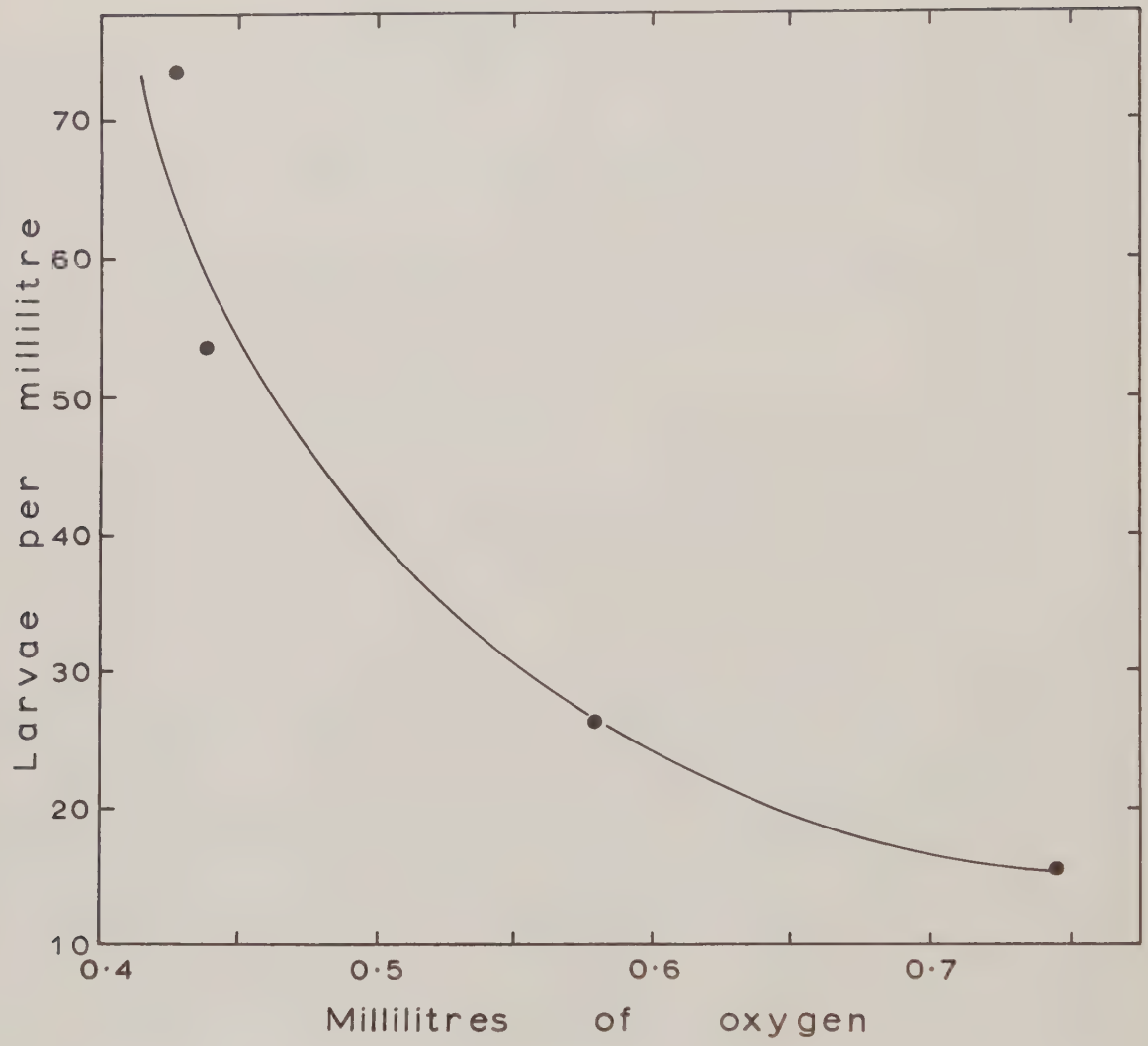

Fig. 22. *The calculated oxygen consumption of* 10 000 *larvae, related to the density of the larvae. Duration of experiment* 21 *hours.*

In the second experiment, similar bottles were set up with filtered water and sufficient *Isochrysis* to give about 110 cells/µl. Approximately equal numbers of larvae of a mean size of 219µ were added to each bottle; counts made at the end on the individual bottles showed that the densities varied between 24 and 30 per ml. Individual bottles were withdrawn after various time intervals, and the oxygen content and larval numbers were estimated. From these data, the oxygen consumed by 10 000 larvae has been calculated for each bottle and this is plotted in Figure 23A against time. From this curve, which clearly shows the declining rate of oxygen consumption with the decrease in available oxygen, consumption has been calculated for successive five-hour intervals from the beginning of the experiments; in Figure 23B these are related to the mean concentration in the middle of each period. This demonstrates how closely consumption was related to oxygen concentration.

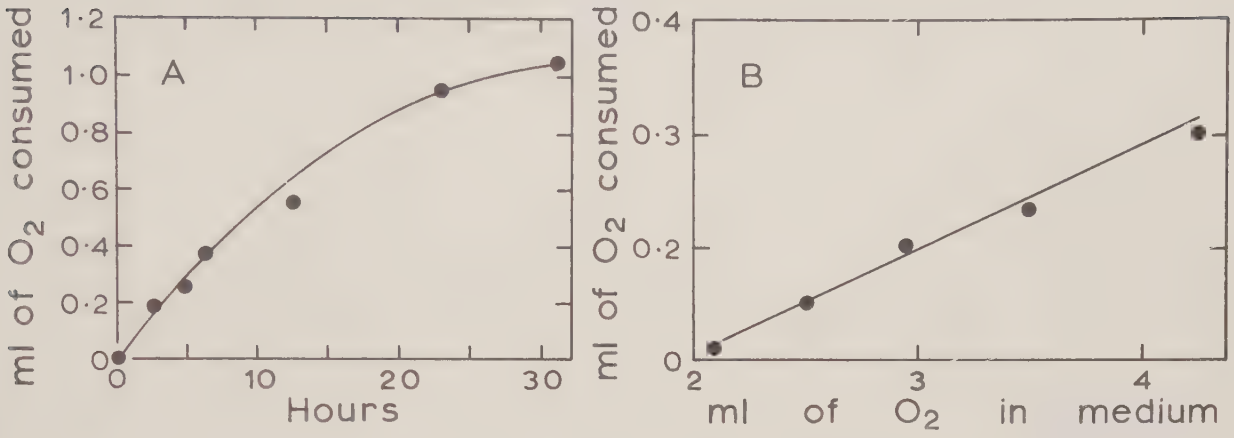

Fig. 23. *A: The calculated oxygen consumption of* 10 000 *larvae confined in glass bottles. B: The consumption in successive five-hour periods* (*ordinate*) *calculated from A, related to the mean oxygen concentration in the water during that period* (*abscissa*).

Full-salinity sea water at 22°C and normal pressure has an oxygen content of 5·2 ml/litre (Harvey, 1955). From an extrapolation of Figure 23B, 10 000 larvae will consume 1·5-2·0 ml 0_2 in five hours or 7·2-9·6 ml/day at this oxygen concentration. Zeuthen (1947) estimated the oxygen consumption of individual larvae of *Mytilus edulis*, *Zirphaea cristata* and *Mya arenaria* at about 1×10^{-3} µl 0_2/hour, which is equivalent to 2·4 ml/day/10 000 larvae. In the rearing technique described in this paper, larvae of a similar size will be at a density of about 1 100 per litre or 10 000 in 9 litres, and will therefore have $9 \times 5{\cdot}2$ or 46·8 ml of oxygen available. Assuming that there was no gaseous exchange, the estimated consumption data suggest that at the most the larvae would use only 20% of the available oxygen in 24 hours. Occasional observations on the oxygen content of the large-scale experiments showed that they were always close to saturation.

LIGHT

All the large-scale experiments reported in this paper were made in a room without windows. The only light reaching the larvae was therefore from the laboratory lighting when people were working in the room. Despite some casual observations that directional light will, on occasions, stimulate larvae to swim, comparative small-scale experiments have not shown any substantial difference between growth in the light and in the dark (see p. 45). Three comparative experiments have been made in which growth and spatfall in paired 75-litre polythene bins were compared in the light and the dark. In the first experiment the standard procedure was followed, except that one of the pair was fitted with an opaque lid and the other had a transparent plastic lid, above which hung a continuously lit 100-W filament light bulb. The larvae grew equally well in both bins, and on day 9 two strings of five mussel shells were hung in each and sixty to seventy mussel shells scattered on the bottom. The following counts of spat were recorded on the shells:

	Daily spatfall on 10 suspended shells	
	Light	Dark
Day 10	30	occasional
11	71	13
12	33	28
13	14	59
14	6	66
Total spat on shells on bottom of tank	8 500	4 300

The experiment was stopped on day 14, when 60 000 large larvae were still present in both vessels.

In the second experiment 250 000 larvae were cultured together in the dark for the first 48 hours after release, during which time they grew from 195 to 206μ. After this they were subdivided into three lots and reared for the next ten days in 50 litres of water in 75-litre bins. One batch was lit continuously, one was in the dark, and the third was only lit until day 7, when they had a mean size of 277μ. On day 8 two strings of five mussel shells were hung in each tank, and on the next day 36 shells were placed on the bottom of each tank. The following counts of spat were recorded on the shells:

	Daily spatfall on 10 suspended shells		
	Light	Dark	Light on days 2-7
Day 9	7	0	8
10	6	2	0
11	107	1	20
12	171	14	15
Total spat on shells on bottom of tank	2 660	420	396

The growth rate was very good and about the same in all three bins, and when the experiment finished good numbers of eyed larvae were present in all three.

In the third experiment a batch of larvae was cultured in two 75-litre bins in the dark until day 9 when they were pooled and then again split between two 75-litre bins. At this time their mean size was 268μ and none had developed eye spots. The experiment was then continued with one bin in complete darkness while the other had a continuous light from a 60-W bulb shining through the transparent top. Spatfall was recorded daily near the surface on two strings of five mussel shells and at the bottom on ten or eleven frosted glass plates 9 cm square, which were examined at intervals. The further growth of the larvae and the proportion eyed were very similar in both bins, but again spatfall was earlier and more prolonged in the light (Figure 24). The sum totals of the spatfall recorded during the experiment were:

	Light	Dark
10 shells at surface	4 661	989
10 plates on bottom	9 598	5 698

From these three experiments it appears that while light has little effect on growth, it does have a stimulating effect on spatfall. This relationship warrants further investigations and will probably result in a modification of the standard rearing technique.

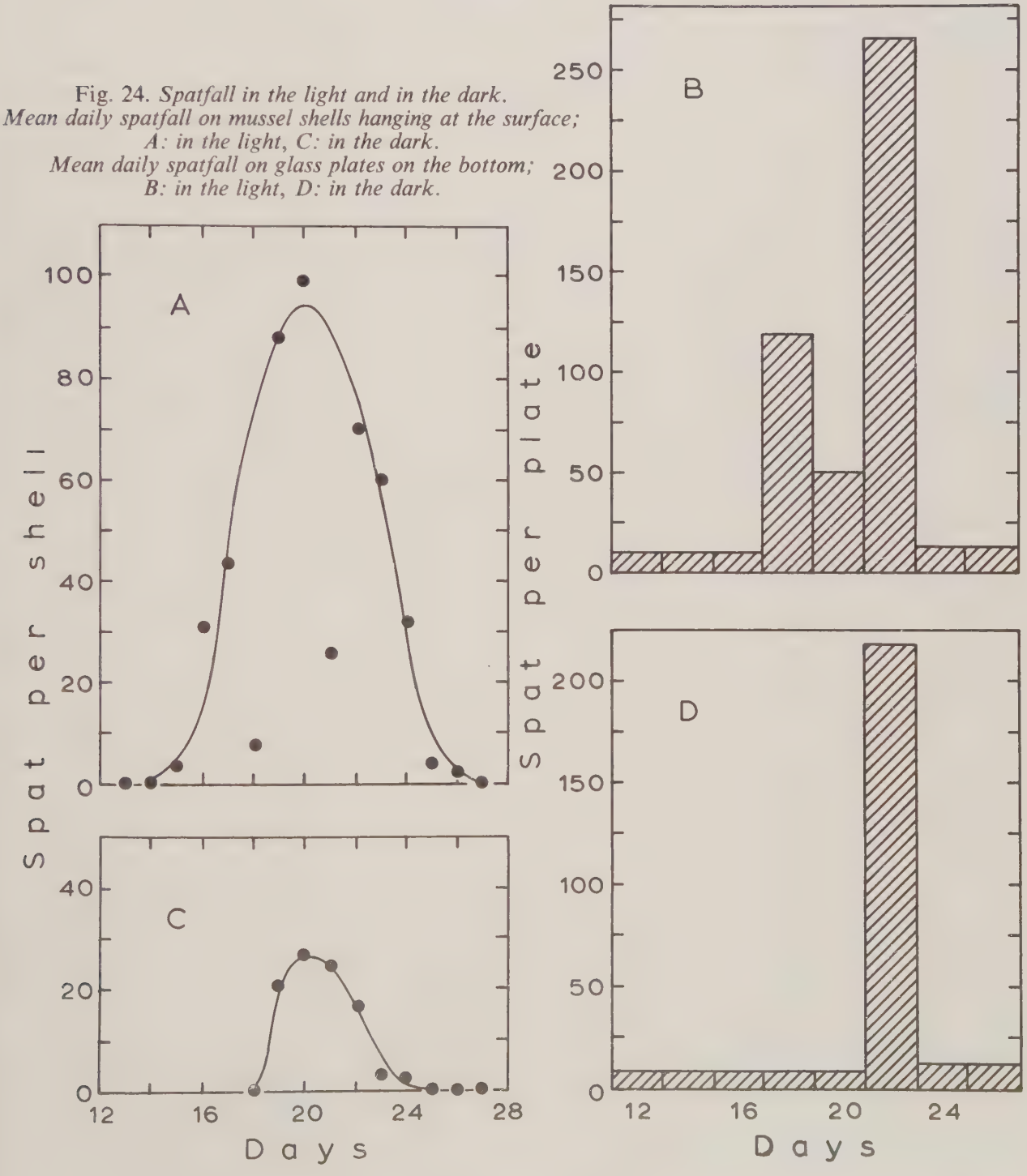

Fig. 24. *Spatfall in the light and in the dark.*
Mean daily spatfall on mussel shells hanging at the surface;
A: in the light, C: in the dark.
Mean daily spatfall on glass plates on the bottom;
B: in the light, D: in the dark.

OYSTER EXTRACT

It was shown by Cole and Knight-Jones (1949) that oyster spat prefer to settle on surfaces bearing live oysters, and similar studies with other marine invertebrates have shown this to be a widespread phenomenon. Later Crisp and Meadows (1962) demonstrated that barnacle larvae were stimulated to settle on plates which had been treated with aqueous extracts of adult barnacles. The experiments reported below show that a similar phenomenon occurs in *Ostrea edulis*, and it is possible that this technique would result in improving the number and distribution of spat on the collectors.

An aqueous extract of oyster meat was prepared by homogenising the meat from an oyster in about 100 ml of sea water. The mixture was clarified by first filtering through a cloth and then centrifuging to remove most of the particulate material. Finally, the extract was painted onto frosted glass plates 9 cm square which were dried either in the laboratory or in a cool oven. When settlement was about to begin 10 to 12 plates were distributed on the bottom of the bin, and after various periods the plates were removed and the number of spat counted. It was found to be advisable for the spat to be allowed to grow on the plates for one or two days after they had removed from the larval culture. This made it possible to distinguish clearly those larvae which had completed metamorphosis from those which were moribund.

Table 15. *The settlement of spat on frosted-glass plates on the bottom of the 75-litre polythene bins. Some of the plates had been painted with an aqueous extract of oyster meat, while untreated plates served as controls. The figures in parentheses showthe number of plates in each treatment. Experiments 1, 2 and 3 were with different batches of larvae. Experiments 4, 5 and 6 were with the same batch of larvae which had been divided between three bins treated in the following manner:—*

Experiment 4. In dark and with a bundle of P.V.C. collectors.
Experiment 5. In continuous light and with a bundle of P.V.C. collectors.
Experiment 6. In continuous light and no P.V.C. collectors.

Experiment	Days since liberation of larvae	Number of days plates exposed to settlement	Average number of spat on		Ratio of spat on untreated : treated plates
			Untreated plate	Treated plate	
1	11–13	2	33·5(6)	62·0(4)	1 : 1·85
2		6	78·2(5)	33·0(5)	1 : 0·42
		2	52·6(5)	423·2(5)	1 : 8·04
3		10	129·2(5)	31·6(5)	1 : 0·24
		2	117·8(6)	839·7(4)	1 : 7·13
4	9–12	3	5·0(6)	20·9(6)	1 : 4·18
	12–13	1	40·0(6)	78·7(7)	1 : 1·97
	13–15	2	111·4(8)	315·0(4)	1 : 2·83
	15–17	2	11·7(6)	54·2(6)	1 : 4·63
5	9–12	3	22·5(6)	87·0(6)	1 : 3·87
	12–13	1	63·5(6)	448·5(6)	1 : 7·06
	13–15	2	269·8(6)	318·5(6)	1 : 1·18
	15–17	2	33·2(6)	126·3(6)	1 : 3·80
6	9–12	3	40·9(6)	33·5(6)	1 : 0·82
	12–13	1	8·7(6)	80·3(6)	1 : 9·23
	13–15	2	316·8(6)	695·6(6)	1 : 2·20
	15–17	2	22·3(6)	31·5(6)	1 : 1·41

The results with four different broods of larvae are shown in Table 15. The average ratio of the spatfall on untreated:treated plate was 1:3·58. In only three out of the seventeen sets of observations did more settle on the untreated plates. In one of these the ratio was close to 1 and in the other two instances the plates were exposed to settlement for longer than usual. It may be that this longer period allowed the attractive substance to decay; perhaps, in addition, bacterial growth, stimulated by the organic substances in the extract, made the surfaces unattractive to the larvae. Experiments 4, 5 and 6, which were replicate cultures from the same batch of larvae, show that the treated plates retained their attractive quality whether the experiment was in the light or the dark, and whether the usual plastic collector was present or not.

ACKNOWLEDGEMENTS

Work of the type described in this paper involves experience in a number of fields, and I wish to take this opportunity of thanking the specialists in numerous facets of marine biology who have freely given advice. My thanks are particularly due to the following colleagues: Mr. Morris Jones for the construction of many pieces of equipment, Mr. D. R. Williams, who has assisted with the work on oyster rearing for many years, and Mr. B. E. Spencer, who carried out many of the experiments reported in this paper.

SUMMARY

This paper describes a technique for rearing oyster larvae in batches of 100 000 and gives the results of a series of standard experiments made in 1963 and 1964. The methods of handling the breeding stock and culturing the algal food are described. The results are given of a number of other experiments which are relevant to the techniques adopted, and the reasons for their adoption are discussed. Further lines of investigation are considered.

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